University of Dundee

MASTER OF SCIENCE

Development of an inducible psoriasis-like in vivo model

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Masters of Science by Research

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Abbreviations

5,6 – DiHETE	5,6-Dihydroxy-7,9-Trans-11,14-Cis-Eicosatetraenoic Acid
8 – MOP	8-Methoxypsoralen
AF-2	Activating Function 2
AhR	Aryl Hydrocarbon Receptor
AP1	Activator Protein 1
APS	Ammonium Persulfate
BAX	Bcl-2–Associated X Protein
BCA	Bicinchoninic Acid
BCL – XL	B-Cell Lymphoma-Extra Large
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
cDNA	Complementary Deoxyribonucleic Acid
CHAPS	3-(3-Cholamidopropyl)Dimethylammonio-1-Propanesulfonate
CO_2	Carbon Dioxide
CYP1A1	Cytochrome P450, Family 1, Subfamily A, Polypeptide 1
CXCL1	Chemokine (C-X-C Motif) Ligand 1
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DPX	Di-N-Butyle Phthalate In Xylene

DR1	Direct Repeat 1
DXE/XRE	Diverse Sequence Xenobiotic Responsive Element/Xenobiotic Responsive Element
ECL	Electrochemiluminescence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FCS	Fetal Calf Serum
FGF-10	Fibroblast Growth Factor 10
FLG	Filaggrin
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GAS-Like	Gamma-Interferon Activation Site-Like
GC Box	Guanine Cytosine Box
GM-CSF	Granulocyte-macrophage Colony Stimulating Factor
GWAS	Genome Wide Association Study
H&E	Haematoxylin And Eosin
HB-EGF	Heparin-Binding EGF-Like Growth Factor
HDL	High-Density Lipoprotein
HGF	Hepatocyte Growth Factor
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HLA-C	Human Leukocyte Antigen C
HRP	Horseradish Peroxidase
H_2O_2	Hydrogen Peroxide
ICAM-1	Inter-Cellular Adhesion Molecule 1
IDEC	Inflammatory Dendritic Epidermal Cells
IFI27	Interferon Alpha-Inducible Protein 27
IFN	Interferon

IFN-A	Interferon Alpha
IFN-Γ	Interferon Gamma
IκB	Inhibitor Of Kappa B
IKKB	Inhibitor Of Nuclear Factor Kappa B Kinase Beta Subunit
IL	Interleukin
IL – 1	Interleukin - 1
IL – 1β	Interleukin – 1β
IL – 2	Interleukin - 2
IL – 4	Interleukin - 4
IL – 5	Interleukin - 5
IL – 6	Interleukin - 6
IL – 8	Interleukin - 8
IL – 10	Interleukin - 10
IL – 12	Interleukin - 12
IL – 12B	Interleukin – 12 Subunit Beta
IL – 12R	Interleukin – 12 Receptor
IL – 13	Interleukin - 13
IL – 17A	Interleukin – 17 A
IL – 17F	Interleukin – 17 F
IL – 18	Interleukin 18
IL – 20	Interleukin - 20
IL – 22	Interleukin - 22
IL – 23	Interleukin - 23
IL – 23A	Interleukin – 23 Subunit Alpha
IL – 26	Interleukin - 26
IL – F5	Interleukin – F5

I3C	Indole-3-Carbinol
IMS	Industrial Methylated Spirit
iNOS	Inducible Nitric Oxide Synthase
JAK 2	Janus Kinase Containing Receptor 2
K1	Keratin 1
K10	Keratin 10
KCl	Potassium Chloride
kDa	Kilo Dalton
KGF-2	Keratinocyte Growth Factor 2
LCE	Late Cornified Envelope
LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharide
LXA4	15-Epi-Lipoxin A4
MAPK	Mitogen-Activated Protein Kinase
MCL – 1	Induced Myeloid Leukemia Cell Differentiation Protein
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
N_2	Nitrogen
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NK	Natural Killer Cell
NLR	Nucleotide-binding Domain, Leucine-rich Repeat-containing
O.C.T	Optimal Cutting Temperature Compound
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCDH21	Protocadherin 21

PCR	Polymerase Chain Reaction
pDCs	Plasmacytoid Dendritic Cell
PEG	Polyethylene Glycol
PPAR	Peroxisome Proliferator Activator Receptor
PPRE	Peroxisome Proliferator Response Element
PTK6	Protein Tyrosine Kinase 6
PUVA	Psoralen + UVA
RNA	Ribonucleic Acid
RNase	Ribonuclease
RXR	Retinoid X Receptor
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SDS–PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2
SPR	Small Proline-rich Protein
STAT	Signal Transducers And Activators Of Transcription
TAD	Transactivating Domain
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween 20
T_c	T Cytotoxic Cell
TGFα	Transforming Growth Factor A
Th	T Helper Cell
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TNFα	Tumour Necrosis Factor A
TNFAIP3	Tumor Necrosis Factor, Alpha-Induced Protein 3

TNIP1	TNFAIP3-Interacting Protein 1
TRIS-HCL	Tris (Hydroxymethyl) Aminomethane – Hydrochloride
Tyr705	Tyrosine 705
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
α	alpha
β	beta
γ	gamma
δ	delta

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Finally I would like to dedicate this thesis to all my friends, family and colleagues who have supported me throughout the research and writing of my thesis, particularly during the later stages of the write-up.

Declaration

I declare that the following thesis is based on the results of experiments carried out by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the relevant researchers or their publications. This dissertation has not been accepted or previously submitted in whole, or in part, for a higher degree.

Louise Reilly July 2011

Supervisors Declaration

I certify that Louise Reilly has spent the equivalent of at least six terms in research work in the Centre for Oncology and Molecular Medicine, Division of Medical Sciences, Ninewells Hospital, University of Dundee and that she has fulfilled the conditions of the Ordinance General No. 39 of the University of Dundee and is qualified to submit the accompanying thesis in the application for the degree of Master of Science.

Dr John Foerster

Senior Clinical Fellow

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July 2011

Abstract

Psoriasis is a common chronic inflammatory skin condition. Several key mediators have been identified to play a role in the pathogenesis of this disease including PPARδ and STAT3. PPARδ is a member of the Peroxisome Proliferator Activator Receptor family and has been implicated in psoriasis, as supported by microarray and immunohistochemical data. Based on these data, we sought to generate an in vivo model that overexpresses PPARδ specifically in the epidermis. A model was available that we sought to characterise. This model, following induction with a specific PPARδ ligand, results in a psoriasis-like skin phenotype, accompanied by immune system activation and gene dysregulation. In addition, STAT3 is highly activated in this animal model in the suprabasal layer of murine skin, which is an important feature of psoriasis. The inhibition of STAT3 inhibits phenotype development in this model. In addition, in this model, interferon response genes are downregulated, which contrasts with human psoriasis. However this downregulation is partially reversed following treatment with WP1066 as measured by the expression of IFI27, suggesting that activation of STAT3 is at least partially responsible for IFN target gene repression. This data show that epidermis-specific overexpression of PPARδ results in a psoriasis-like phenotype, suggesting a central role for PPARδ in psoriasis. STAT3 is also highly activated in this model, supporting the current evidence that STAT3 plays a role in psoriatic disease. Together, this suggests that STAT3 activation occurs downstream of PPARδ activation and indicates that inhibition of PPARδ may be effective for the treatment of psoriasis.

Chapter 1: Introduction

1. Introduction

Psoriasis is a common inflammatory skin disease, affecting around 25 million people in Europe and America (Lowes et al 2007, Westergaard et al 2003), and is one of the most common immune-mediated skin diseases in adults. In order to develop novel therapies for the disease it is vital to understand molecular events underlying its pathogenesis.

To date, there have been myriad studies utilising different approaches to uncover the mechanisms underlying psoriasis disease development, including cell culture (*in vitro*), animal models (*in vivo*), and genetic studies of patients. This has identified a number of different genes and pathways that may contribute to disease progression, two of which will be discussed in detail in this thesis, Peroxisome Proliferator Activator Receptor δ (PPAR δ), and Signal Transducers and Activators of Transcription 3 (STAT3).

1.1 Skin Physiology: Structure And Function

The skin is the body's outermost defence against invading pathogens and microbes. The skin plays a vital role in the overall homeostasis of the body via the excretion of salts, water and organic wastes, maintenance of normal body temperature, storage of lipids and synthesis of vitamin D_3. It is a highly complex structure, composed of several different cells types, which form into different layers. There are three distinct layers, the epidermis, the dermis and the subcutaneous layer.

1.1.1 The Epidermis

The epidermis is the uppermost of the three layers in the skin and can be further subdivided into 5 layers, the basal layer, spinous layer, granular layer, stratum lucidium and stratum corneum. The innermost layer is the basal layer and is the only layer that has the capacity for DNA synthesis and mitosis (Fuchs 1990). Cells from the basal layer, following an as yet unidentified trigger, commit to terminal differentiation and begin their journey to the surface of the skin. During this journey, the basal cell will undergo changes that ultimately result in the formation of dead, flattened, and enucleated cells that are expendable and are continually replaced by cells from the lower layers undergoing the same process.

The basal layer is the only layer where proliferating keratinocytes are located, which adhere to the underlying basement membrane (Pincelli and Marconi 2010). Cells in the basal layer detach from the basement membrane at set intervals and withdraw from the cell cycle and initiate terminal differentiation. Following commitment to terminal differentiation, keratinocytes progress through three different stages, which form the different layers of the skin, the spinous, granular and finally the stratum corneum (Pincelli and Marconi 2010). The molecular mechanisms that drive this process are complex and involve many different genes. This process is still poorly understood (Blanpain and Fuchs 2009).

The cells in the basal layer include keratinocyte stem cells. There are 3 different types of keratinocyte stem cells present in the epidermis which are found at different locations therein: interfollicular epidermis, hair follicles, and sebaceous glands (Pincelli and Marconi 2010). The interfollicular stem cells are principally involved with the

regeneration of the epidermis, which is dependent on the proliferation of these cells. Stem cells actually divide relatively infrequently. When they do divide, they produce a stem cell daughter cell and a rapidly dividing non-stem committed progenitor cell, termed a transit amplifying cell (Pincelli and Marconi 2010). Transit amplifying cells only divide a relatively small number of times before they commit to terminal differentiation in the suprabasal layer.

The intracellular cytoskeleton of basal cells is composed of a dispersed but extensive network of keratin filaments, mainly keratin 5 and keratin 14 (Fuchs 1990). Connecting these cells are structures, which are also present in the spinous layer, called desmosomes. These are calcium-activated membrane junctions that help connect the cells in the basal layer to form a three-dimensional lattice (Franke et al 1987). As the cells migrate upwards towards the surface, different genes that enable differentiation are activated. When cells reach the spinous layer, they are principally involved in the synthesis of two different keratins, mainly K1 and K10, which form cytoskeletal filaments that aggregate into thin bundles (Eichner et al 1986). It is in the spinous layer that keratinocytes begin to synthesise involucrin, an envelope protein that is deposited on the inner surface of the plasma membrane of each cell (Rice and Green 1979). Synthesised at the same time are membrane-coating granules, which fuse with the plasma membrane and release lipids into the intercellular space of granular and stratum corneum cells (Fuchs 1990, Swartzendruber et al 1989).

Once cells from the spinous layer migrate into the granular layer, production of keratin and envelope proteins is ceased and cells begin to produce filaggrin. Filaggrin is a crucial component of the cornified layer and helps keratin filaments aggregate (McGrath and Uitto 2008). Another important protein synthesised in this layer is

loricrin, which is a major component of the cornified layer, as set forth below (Mehrel et al 1990). The differentiating cells present in this layer are permeable and allow an influx of calcium that in turn causes the activation of transglutaminases, which are involved in the covalent cross-linking of the proteins produced here to form high-molecular weight components that attach to the membrane lipids (Hitomi 2005). There are several transglutaminases expressed in the skin, mainly 1, 3 and 5. As previously mentioned, they are regulated by a rise in intracellular calcium. When this occurs, it causes transglutaminases to become activated, resulting in them crosslinking involucrin and other desmosomal proteins such as envoplakin and periplakin (Hitomi 2005). This results in the production of a mono-molecular layer that forms a scaffold. This scaffold can be utilised as a platform for the addition of other reinforcement proteins, such as loricin and small proline-rich proteins (SPRs), which can be cross-linked to form homo- and heterodimers. Loricrin constitutes around 80% of the cornified cell envelope (Kalinin et al 2001), which is complexed with varying amounts of small proline-rich proteins.

During this process, other cell components and organelles are degraded. The keratin intermediate filaments remain, which at this stage of epidermal differentiation are mainly K1 and K10 (Kalinin et al 2001). These are also cross-linked to the cornified cell envelope, desmoplakin and envoplakin remnants and involucrin, loricrin and SPRs. Thus, the resultant dead cornified cell contains mostly bundled intermediate filaments that are covalently attached to and enclosed within the cell envelope (Kalinin et al 2001). This results in a barrier that is durable but also flexible, and is able to provide mechanical and water-permeability functions necessary for the survival of the body.

1.1.2 The Dermis

The dermis is located between the epidermis and the subcutaneous layer and is composed is connective tissue that adds to the overall stability of the skin. The dermis can be defined as two distinct layers, the papillary dermis and the reticular dermis. The papillary dermis is the most superficial of the two layers, and is approximately 300-400μm deep (Sorrell and Caplan 2004). The upper section of this layer forms dermal projections containing components that help maintain the epidermis. The papillary dermis is thinner than the reticular dermis, and is composed predominantly of loose, poorly organised collagen fibre bundles, as well as elastic fibres. The major collagens found here are type I and type III. In addition to these fibrous collagens, non-fibrillar collagens, such as type XII and XVI are also present in the papillary dermis (Berthod et al 1997, Walchli et al 1994).

A vascular plexus demarcates the lower limit of the papillary dermis (Sorrell and Caplan 2004). The reticular dermis extends from this plexus down until it reaches another plexus that marks the boundary between the dermis and the subcutaneous layer. The reticular layer is more dense than the papillary dermis, composed of thick, well-organised collagen fibre bundles, mainly consisting of a low ratio of type III collagen to type I. Proteoglycans, such as decorin, are dispersed between the collagen fibres in this layer, whereas versican is expressed in highly quantities in the elastic fibres of the reticular dermis (Zimmermann et al 1994). Non-fibrillar collagens are also expressed in the reticular dermis, most notably, collagen type IV (Sorrell and Caplan 2004).

The predominant cell type present in the dermis are fibroblasts. They are involved in the regulation of the proliferation of keratinocytes in culture and also play roles in wound repair. Factors produced by fibroblasts, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), FGF-10 (KGF-2), parathyroid-hormone-related protein, hepatocyte growth factor/scatter factor (HGF/SF), epidermal growth factor (EGF) and IL-6, are all involved in modulating the activity of keratinocytes in response to various stimuli, such as injury (Sorrell and Caplan 2004). The papillary dermis is more metabolically active and contains more fibroblasts than the reticular dermis (Harper and Grove 1979), which one would expect since it is the closest to the epidermis and thus supplies the keratinocytes therein with the stimulatory or inhibitory signals that govern their proliferation and differentiation.

1.1.3 The subcutaneous layer

The subcutaneous layer is the lowermost layer of the skin. Although the subcutaneous layer is not technically part of the skin, connective tissue fibres from the reticular layer are extensively interwoven with those of the subcutaneous layer. The superficial region of this layer contains large arteries and veins that supply the dermis and, by diffusion, the epidermis with nutrients essential for the maintenance and homeostasis of the skin. It is predominantly composed of loose connective tissue and adipose tissue. The subcutaneous layer has an important role in the regulation of skin temperature as well as contributing to the control of body temperature. The thickness of this layer varies from person to person. The subcutaneous layer also plays a role in protection of the internal organs and skeleton through the storage of fat deposits.

1.1.4 The Immunology Of The Skin

The skin serves as a very important component of the body's immune system, with the epidermal barrier involved in innate immunity where the body defends itself against invading pathogens by the mobilisation of immune cells in a non-specific manner. It is vitally important that the immune response exhibited by the skin in response to pathogens is suitable. If the response is inadequate it could lead to serious infection or the development of tumours, whereas if it is excessive it can cause chronic inflammation and autoimmunity (Nestle et al 2009). There is a wealth of immune cells found the skin. In the epidermis, Langerhans cells, other dendritic cells and T cells are abundant, whereas in the dermis, innate immune cells, $CD4^+$ T helper cells, natural killer T cells, macrophages, mast cells and fibroblasts are all present (Figure 1.1).

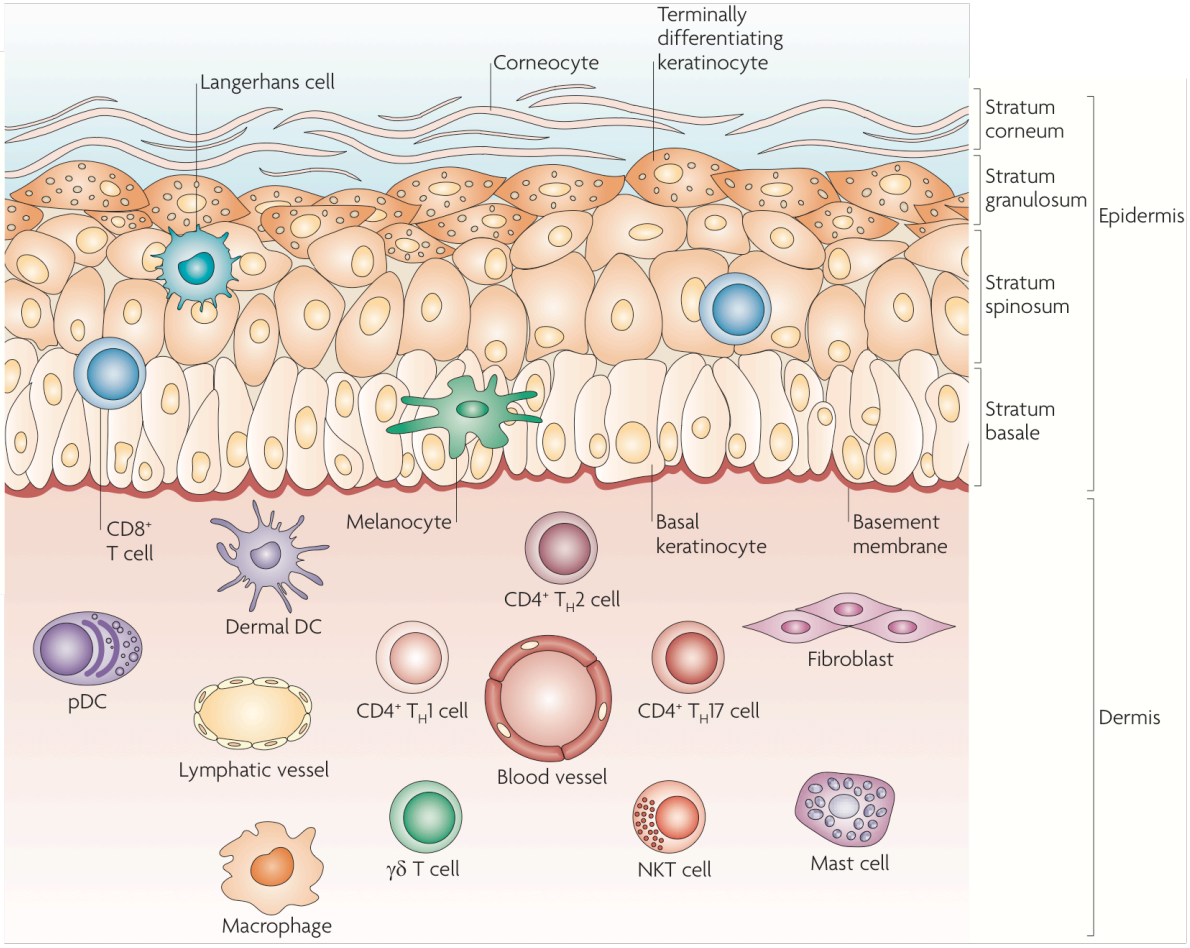

Figure 1.1: The Skin Contains Many Different Immune Cells. *The skin is a highly complex structure tasked with defending the body against invading pathogens as well as maintaining body temperature and providing sensory information to the central nervous system. There are many different immune cells present in normal healthy skin, in the epidermis, Langerhans cell and $CD8^+$ T cells are found. In the dermis, there are a wider variety of immune cells present, such as plasmacytoid dendritic cells, $CD4^+$ T_H1 cells, $CD4^+$ T_H17, fibroblasts, macrophages, natural killer T cells, mast cells, $CD4^+$ T_H2 and dermal dendritic cells. All these cells play a pivotal role in maintaining skin immune homeostasis. Modified from (Nestle et al 2009).*

As previously discussed, keratinocytes are the major cell type in the epidermis. These cells are also capable of sensing pathogens, and help to differentiate between harmless organisms and harmful pathogens (Nestle et al 2009). Utilising various receptors expressed on their membrane, keratinocytes can recognise microbial products via recognition of conserved elements contained within them termed pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the most well known of these receptors, and once activated, contribute to the T_H1-type immune response via the secretion of type I interferons (IFNs) (Miller and Modlin 2007). In addition to TLRs, keratinocytes also express proteins that are the products of the nucleotide-binding domain, leucine-rich repeat-containing (NLR) gene family, which are capable of recognising PAMPs. They are able to form a complex with apoptosis associated speck-like protein and pro-caspase 1 that is termed the inflammasome (Martinon et al 2009). The formation of the inflammasome leads to the activation of caspase I, which cleaves pro-IL1β and pro-IL18, resulting in the production of pro-inflammatory cytokines. Keratinocytes produced antimicrobial peptides, particularly β-defensin and cathelicidins, which can be increased in response to skin infection by T cell-derived cytokines, such as IL-17A and IL-22, which are produced by T_H17 cells (Liang et al 2006), thereby providing a link between keratinocytes and adaptive immune cells. Keratinocytes secrete a milieu of cytokines, IL-1, IL-6, IL-10, IL-18 and TNF, which lead to the activation of T_H cells and dendritic cells as well as stimulating B cell maturation (Nestle et al 2009). Expression of various molecules on their cell surface allows keratinocytes to attract effector T cells and neutrophils and regulate the trafficking of Langerhans cell precursors. Although there are many specialised immune cells present in the skin, keratinocytes play a central role to the recruitment of many of these cells via the secretion of various cytokines and expression of molecular markers.

Dendritic cells constitute a large number of the immune cells that are present in the skin. Langerhans cells are predominantly expressed in the epidermis, particularly the suprabasal layers (Nestle et al 2009). Langerhans cells are the first line of defence against invading microbial antigens. They contain a unique cytoplasmic organelle called the Birbeck granule (Nestle et al 2009). Langerhans cells' primary function is to take up invading antigens and process them for presentation to effector T cells. They have also been shown to induce differentiation of T_H2 cells as well as prime and cross-prime naïve $CD8^+$ T cells (Klechevsky et al 2008). Inflammatory dendritic epidermal cells (IDEC) are also present in the epidermis and play a role in pro-inflammatory allergic reactions, due to the increased expression of the high affinity Fc receptor for IgE. As well as epidermal dendritic cells, there are a number of dermal dendritic cells found in the skin. Dermal dendritic cells are capable of inducing T cell proliferation (Fukunaga et al 2008) and also secrete cytokines and chemokines to generate a cytokine network, which can be beneficial as it eradicates infectious agents but if unchecked then can contribute to a unnecessary inflammatory reaction (Nestle et al 2009). There are subtypes of dermal dendritic cells that are capable of producing either TNF or iNOS. Another subset of dendritic cells, termed plasmacytoid dendritic cells are rare in healthy skin, but are thought to play an important role in psoriasis. Finally, macrophages, which are also present in the dermis, are sessile (Nestle et al 2009). Under inflammatory conditions, these cells migrate to the lymph nodes (van Furth et al 1985) and aid in the eradication of invading microbes. There is a wealth of dendritic cells present in the skin that all contribute to the immune homeostasis of the skin.

The most prevalent immune mediators present in the skin are T cells. In normal healthy skin, there can be as many as 2×10^{10} T cells (Clark et al 2006), which is more than is present in the blood. Epidermal T cells are found in the basal and suprabasal layers of

the epidermis, usually in close proximity with Langerhans cells. $CD4^+$ T_H cells, of which there are 3 main types, T_H1, T_H2 and T_H17, are found in skin during various inflammatory diseases (Nestle et al 2009). The different subsets of T cells have different roles in inflammatory responses, with T_H1 cell responses contributing to autoimmunity and immune-mediated pathologies, whereas T_H2 cell responses are associated with allergic disease. T_H17 has been shown to be involved in both pathways, and is essential for first line defence against various fungal and bacterial infections (Weaver et al 2007). Like the other immune cells present in the skin, T cells serve as immune sentinels with a role in homeostasis.

1.2 Psoriasis

Psoriasis is an inflammatory skin disease that affects around 1-2% of the population. It appears to be most common in people of Caucasian descent. The prevalence of psoriasis can be affected by latitude, which is presumed to be due to the fact that sunlight has a beneficial effect on the disease (Griffiths and Barker 2007). It can occur at any age, the estimated mean age of onset is 33 years of age, with 75% of cases occurring before the age of 46. In a subgroup of patients, psoriasis also causes arthritis (Lowes et al 2007). Broadly, psoriasis is comparable to other immune-mediated disorders such as Crohn's disease, rheumatoid arthritis, multiple sclerosis and juvenile-onset diabetes in that it involves inappropriately activated effector immune function.

1.2.1 Clinical Features

Psoriasis is a papulosquamous disease with varying morphology, distribution, severity and course (Langley et al 2005). Papulosquamous diseases are characterised by the presence of scaling papules and plaques on the surface of the skin, that are <1cm in diameter and >1cm in diameter respectively. The plaques observed in psoriatic patients are often red, scaly and raised, as shown in figure 1.2. Psoriasis develops or is worsened at sites of mechanical friction, which may explain why plaques are commonly observed on elbows, knees and the scalp. In addition, plaques adopt a symmetrical distribution, the reason for which is unknown. A phenomenon associated with psoriasis, termed Knoeber Phenomenon, is when psoriasis develops at the site of trauma (Langley et al 2005).

Figure 1.2: Clinical Photograph Detailing Plaque Psoriasis. *The left panel shows a patient with psoriasis on the back, note the symmetrical distribution of plaques. The right panel shows a close up photograph of a psoriasis plaque, showing well demarcated edge and silvery scale. Modified from (Langley et al 2005).*

There are 3 principal histological features of psoriasis, epidermal hyperplasia, dilated, prominent blood vessels in the dermis, and an inflammatory infiltrate of leukocytes (Langley et al 2005). Epidermal hyperplasia is due to the increase in proliferation rate of keratinocytes in the interfollicular epidermis (Lowes et al 2007). The epidermal rete pegs become elongated and project down into the dermis as shown in Figure 1.3.

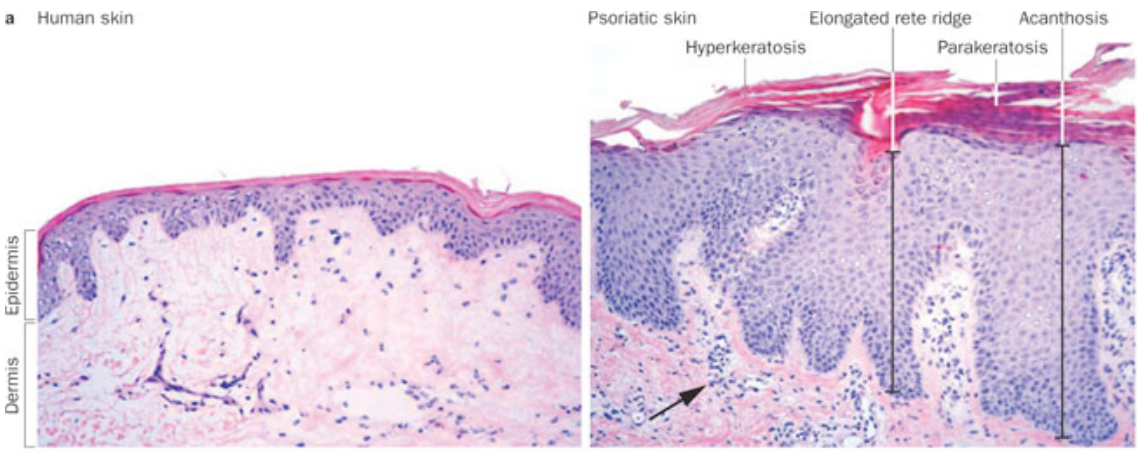

Figure 1.3: H&E Histology Of Human Skin. *The left panel shows normal healthy human skin, whereas the right shows skin affected by psoriasis. Notice the increased thickness of the epidermis with elongation of the rete ridge. The arrow indicates the presence of inflammatory cells in the dermis. Modified from (Wagner et al 2010).*

The differentiation of keratinocytes is extensively altered in psoriasis; they mimic "regenerative maturation", which is an alternative cell differentiation programme that is transiently expressed during wound healing. The hyperplastic epidermal changes observed are associated with the underexpression of markers of keratinocyte differentiation, including keratin 1 and 10 (Griffiths and Barker 2007). Involucrin is upregulated in psoriasis, whereas filaggrin is downregulated (Rashmi et al 2009). In addition, the granular layer, where terminal differentiation initiates, is vastly reduced or

completely absent in psoriatic skin lesions. The stratum corneum forms from incompletely differentiated keratinocytes that aberrantly retain a cell nucleus, a process termed parakeratosis (Lowes et al 2007). As a consequence, scaling and a break in the protective barrier occurs in the skin of psoriasis patients, due to a failure of psoriatic corneocytes to stack normally and secrete lipids that enables them to adhere to one another. Keratinocyte transglutaminase type I (TGase K) is overexpressed in psoriasis and is responsible for the excessive cornification observed in psoriatic plaques (Schroeder et al 1992).

In addition and as mentioned previously, there is an increase in the vascularity of psoriatic lesions and this explains the redness associated with plaques. This is due to angiogenic factors produced by keratinocytes, such as vascular endothelial growth factor (VEGF), which is upregulated in psoriatic plaques (Simonetti et al 2006). Another hallmark of the disease is the presence of neutophils in the small foci of the stratum corneum, (termed Munro's microabscesses) and there is an increase in the number of T cells found in lesions, interspersed between keratinocytes and in larger quantities in the dermis (Krueger and Bowcock 2005). As a result there is a mononuclear inflammatory cell infiltrate dominated by T cells, as shown in Figure 1.4. Neutrophils are also recruited into lesions from blood stores (Lowes et al 2007). Keratinocytes release pro-inflammatory cytokines and chemokines that may produce a chemotactic gradient for the migration of neutrophils in to the epidermis, predominantly IL-8, CXCL1 and S100A7/A8/A9.

A major feature of psoriasis is the aberrant keratinocyte proliferation that is observed, resulting in psoriatic plaques. Stem cells and transit amplifying cells form the major part of proliferating cells in the epidermis, and are functionally altered in psoriasis. A study

investigating the spatial distribution of c-Jun revealed that c-Jun is expressed in the lower proliferation compartment of the epidermal tissue, where it is not expressed in normal healthy skin (Grabe and Neuber 2007, Mehic et al 2005). In addition, keratin expression is altered, with K6 and K16, which are markers of abnormal hyperproliferative conditions, being upregulated in psoriasis (Rashmi et al 2009). In psoriatic skin, keratinocytes take as little as 6-8 days to reach the surface from the basal layer, compared to the approximately 40 days that it takes in normal skin (Bergstresser and Taylor 1977, Roberson and Bowcock 2010). Not only are keratinocytes hyperproliferative, they also appear to be resistant to apoptosis, unlike normal keratinocytes. This may explain why there are increased numbers of keratinocytes in psoriatic skin, not only are they hyperproliferative but they are able to evade apoptosis as well.

1.2.2 Pathogenesis of Psoriasis

Until 1980, psoriasis was believed to be a disease of epidermal keratinocyte proliferation and the cutaneous inflammatory infiltrate was thought to be a secondary event (Griffiths and Barker 2007). It is now widely accepted that psoriasis is an immune mediated disorder. As mentioned previously, there is an infiltrate consisting mainly of T cells, both $CD4^+$ and $CD8^+$ and it is proposed that this precedes the epidermal hyperplasia observed in psoriasis. Th1 cells ($CD4^+$), Tc1 cells ($CD8^+$), and Th17 cells are all present in psoriatic lesions. There are some $CD8^+$ T cells present that are specialized for homing into the epidermis via the expression of $\alpha_E\beta_7$ integrin, which is capable of binding E-cadherin on keratinocytes. In addition, many T cells express CD161 and other killer receptors, which suggests a role for natural killer (NK) T cells (Lowes et al 2007). As previously mentioned, Th1 cells are present in psoriatic skin,

and in conjunction with this, levels of IFN-γ, TNF-α and IL-12, which are Th1 cytokines, are all elevated in psoriasis. The classical Th2 cytokines, IL-4, IL-5 and IL-10 appear to protect against psoriasis, and this is further supported by the success of IL-4 and IL-10 in clinical trials, suggesting that a shift of cytokine milieu from Th1 to Th2 may reverse psoriasis inflammation (Mak et al 2009). By contrast, Th17 cells, that are also present in psoriatic skin, produce IL-17A, IL-17F, IL-22 and IL-26, which are pro-inflammatory cytokines, activating keratinocytes and leading to hyperproliferation and further pro-inflammatory cytokine production. This activation and subsequent recruitment of other immune cells causes an amplification of the inflammatory response. It is becoming apparent that there are a number of different cytokine signalling cascades activated in psoriasis that converge together and result in a psoriatic phenotype.

Dendritic cells also appear to be involved in the pathogenesis of psoriasis. Thus, epidermally located Langerhans cells, dermal dendritic cells and the so-called plasmacytoid dendritic cells (pDCs) are present in increased numbers in psoriatic skin (Griffiths and Barker 2007). pDCs produce IFN-α in response to stimulation via TLR signalling (Mak et al 2009), driving Th1 responses. This is consistent with the view that psoriasis is not only a Th17, but also Th1 mediated disorder. IFN-α produced by pDCs occurs early on in psoriasis development and is transient, reducing as the disease progresses. Langerhans cells, which are expressed in the epidermis, show impaired migration in response to cytokine and allergic stimuli, another key feature of early onset psoriasis. Finally, adhesion molecules that promote leukocyte adherence, are highly expressed in psoriatic skin, predominantly ICAM-1, which is expressed on epidermal keratinocytes and E-selectin which is expressed on dermal capillaries.

The cytokine interactions present in psoriasis are so-called type-1, which describes a relationship between cytokines, such as IL-23 or IL-12, production of IFN-γ and TNF-α production by type 1 T cells and downstream activation of IFN-response genes via STAT1 (Lowes et al 2007). In line with this, the expression of various signal transducers is upregulated in psoriasis, in particular STAT1, STAT3 and NFκB. IFNs have been described to activate STAT1 and TNF or IL-1 pathways, which are capable of activating the NFκB signalling cascade. In addition, IL-20 and IL-22 pathways have been shown to activate both STAT and NFκB pathways. This highly complex set of events is summarised in figure 1.4. NFκB thus has a central downstream role in many cellular processes in psoriasis, with roles in stress responses and keratinocyte proliferation and differentiation (Roberson and Bowcock 2010)

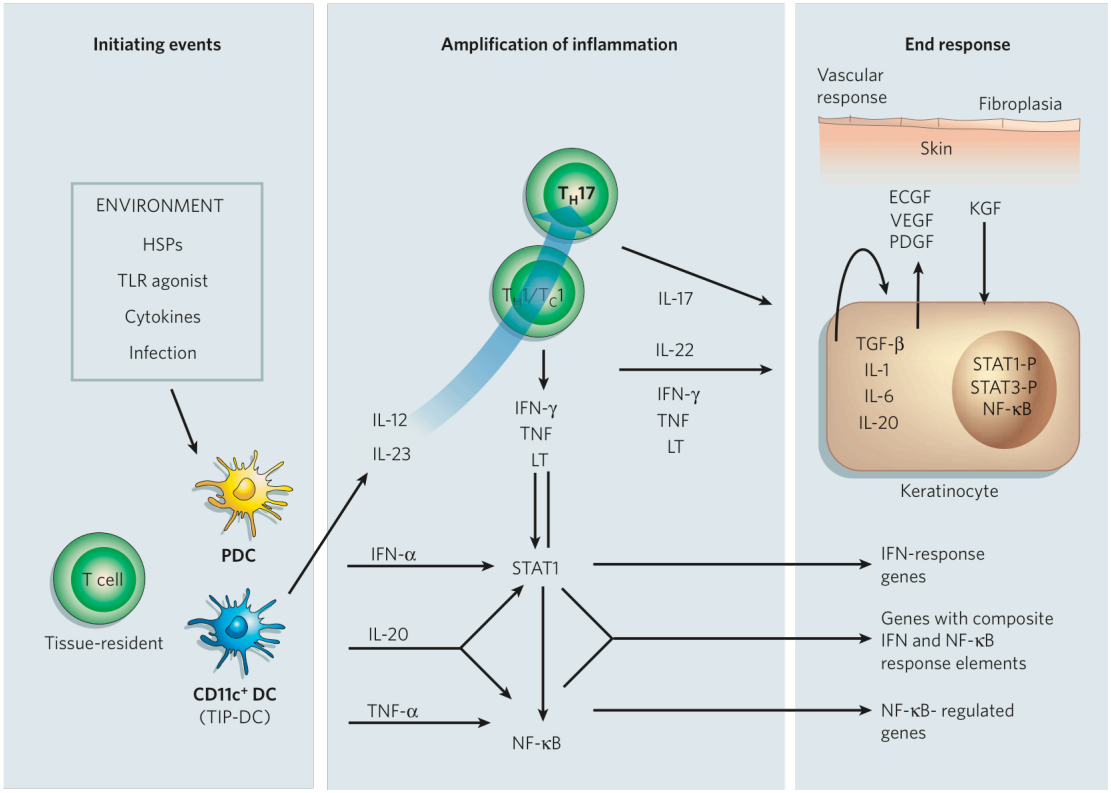

Figure 1.4: Potential Cytokine Signalling Pathways In Psoriasis. *The above diagram demonstrates the possible interactions that occur in psoriatic skin, with the activation of pDCs occurring via environmental factors, heat shock proteins, TLR agonists, cytokines or infection. However this can also occur due to direct interaction with T cells. As a result, various cytokine and chemokine signalling cascades are triggered resulting in the activation of various response genes and elements and subsequent inflammation. (Modified from (Lowes et al 2007).*

1.2.3 Genetic Basis of Psoriasis

Recent advances have highlighted genetic association of genes with psoriasis. Psoriasis is a polygenetic condition. Twin and family studies have revealed a strong genetic component for psoriasis, particularly in those who develop the disease before the age of 40. Approximately 30% of individuals with psoriasis have an affected first degree relative (Griffiths and Barker 2007). In line with this, if both parents and a sibling suffer

from psoriasis, a further child has a 50% chance of developing psoriasis. However that risk reduces considerably if only one sibling is affected. So-called genome wide association scans (GWAS) have been instrumental in discovering new gene loci that may increase an individuals susceptibility to psoriasis and have identified new candidate genes that are consistent with the pathological changes observed.

Due to the strong immune system activation in psoriasis, early genetic studies targeted genes and loci that were known to encode for components of the immune system. MHC class II molecules were investigated first, as it was hypothesised that immune cells in psoriasis were reacting to an unidentified antigen (Roberson and Bowcock 2010). These studies revealed an association between psoriasis and the expression of human leukocyte antigen (HLA) class 1 alleles, with the strongest association being HLA-Cw6 (Roberson and Bowcock 2010, Tiilikainen et al 1980). The frequency of HLA-Cw6 was 46% in psoriasis compared to 7.4% in controls. However, despite the high incidence of this allele in psoriasis cases, the presence of this risk allele is not sufficient for disease development, with the penetrance estimated to be only 10%. Later GWAS studies revealed SNPs in genes present in the MHC class I, which is physically close to the HLAC gene (Liu et al 2008). SNPs that have been found to be associated with psoriasis are correlated with the gene encoding for HLA-Cw6. Located close to the HLA-Cw6 gene is another gene implicated in psoriasis, corneodesmosin. Corneodesmosin has also shown to be associated with psoriasis, with two different alleles of the gene found on known psoriasis-associated haplotypes that also harbour HLA-Cw6 (Nair et al 2000). However, this indicates that corneodesmosin is no more responsible for the psoriasis phenotype than HLA-Cw6.

GWAS studies also confirmed association of IL-12B and IL-12R, both of which had been previously discovered via genetic linkage studies. This study also identified several new candidates, including IL-23A, IL4/IL13, TNFAIP3 (TNFα induced protein 3) and TNIP1 (TNFAIP3 interacting protein) (Elder et al 2010, Nair et al 2008, Nair et al 2009). In addition, there is also a strong association between psoriasis and the region of the late cornified envelope (LCE) genes. Under normal skin conditions, LCE1 and LCE2 are expressed in the skin and are incorporated into the cornified envelope very late in its development (Roberson and Bowcock 2010). However, following tape stripping, which disrupts the cornified layer and leads to epidermal damage, in psoriatic skin, LCE3 expression, is induced (de Cid et al 2009, Elder et al 2010, Roberson and Bowcock 2010). Inappropriate expression of LCE genes could therefore lead to an inappropriate repair response following barrier disruption, due to the repair being insufficiently supported by other LCE genes (de Cid et al 2009, Roberson and Bowcock 2010).

Further associated genes include IL12B and IL23R (Nair et al 2008). The IL12B gene encodes the p40 subunit, which is common to both IL12 and IL23, which are both implicated in the pathogenesis of psoriasis. The IL23R gene on the other hand encodes the subunit for the IL23 receptor, which has also been described to play a role in psoriasis. In a subsequent study several other candidate genes were identified, particularly several involved with NFκB signalling as well as IL23 signalling (Nair et al 2009) including IL23A, which encodes the p19 subunit of IL23. IL23 signalling is necessary for the activation and expansion of Th17 cells, an important feature in the pathogenesis of psoriasis.

Furthermore, the β-defensin cluster on chromosome 8p21 exhibits increased copy numbers in psoriasis patients (Hollox et al 2008, Roberson and Bowcock 2010). β-defensins are small, antibiotic peptides that play a role in the non-specific host defence system. β-defensins are secreted in psoriatic skin by keratinocytes due to the cytokine environment present, a process that can also be enhanced in response to NFκB signalling (Roberson and Bowcock 2010), again suggesting involvement of NFκB in psoriasis pathology. The expression of β-defensin will attract various immune cells, specifically T cells, dendritic cells and neutrophils, all of which are implicated in disease pathogenesis.

Finally, Nair and colleagues identified genes implicated in the modulation of the immune response including IL4 and IL13, which are involved in the humoral response mediated by Th2 cells (Nair et al 2009). Possible dysregulation of these genes may result in a shift from a Th2 response to a Th1 response, thus explaining the marked expansion of Th17 cells observed in psoriatic skin.

Even though GWAS and genetic linkage analysis have elucidated several major signalling pathways involved in psoriasis pathology, it must be stressed that aside from the risk locus in the HLA-C region, genetic effects of all other risk loci are small with effective odds ratios below 1.5, which means that the presence or absence of any one of these risk genes by itself has only a minor effect on the risk of having psoriasis. Importantly, several signalling pathways central to disease pathology do not show any genetic association. Thus, molecular players such as IL-1, STAT3, PPARδ or VEGF have never been demonstrated or successfully replicated (in the case of VEGF). This could be due in part to these molecules signalling downstream of gene products shown to be risk factors.

1.2.4 Therapeutic Approaches

A number of different therapeutic approaches are available for the treatment of psoriasis. Traditional topical therapies, including phototherapy, such as corticosteroids and vitamin D analogues and conventional systemic therapies have been employed for decades, whereas newer biological treatments, targeting specific cytokines, are becoming more widely available.

Topical Therapies

Topical corticosteroids have been a common treatment for psoriasis for decades (Bagel 2009, Mitra and Wu 2010). They are useful for localised plaques, whereas generalised plaques are better treated with phototherapy or a systemic therapy. The efficacy of topical corticosteroids may be attributed to multiple mechanisms of actions that they display, including anti-inflammatory, immunosuppressive and anti-proliferative effects (Mitra and Wu 2010). Although effective, topical and easy to use corticosteroids do carry a risk of developing adverse effects (Horn et al 2010). A number of corticosteroids, ranging from high to low potency are available commercially for treatment, including clobetasol propionate, betamethasone dipropionate and triamicinolone. Other topicals include analogues of vitamin D such as calcipotriol, tacalcitol and maxacalcitol. They have all been demonstrated *in vitro* to have an effect on corneocyte proliferation and differentiation (Mitra and Wu 2010). Calcipotriol, a synthetic analogue of vitamin D, has been shown to have anti-proliferative effects at pharmacologically relevant concentrations, as well as pro-differentiation effects on keratinocytes (Lehmann 2009). Beyond vitamin D analogues, dithranol, coal tar and emollients act broadly and with the exception of emollients, which have been shown to

provide a barrier where it has been lost, have poorly defined specificity.

Systemic Therapies

Systemic therapies for psoriasis, particularly generalised psoriasis, have proved quite effective in treatment. Methotrexate, initially used in the treatment of cancer, was first observed to have a therapeutic benefit in cancer patients suffering from psoriasis (Warren and Griffiths 2008). Methotrexate raises the intracellular levels of adenosine, which may influence leukotriene production, T cells and adhesion molecule expression (Warren and Griffiths 2008) and is also anti-proliferative.

Retinoids are also used in the systemic treatment of psoriasis. Acitretin, a retinoid, is able to activate all subtypes of retinoic acid receptors and as such directly affects keratinocytes via returning their proliferation and differentiation status towards normal levels. Retinoids have also been described to inhibit VEGF, although this is an indirect effect mediated by activator protein 1 (AP1) (Diaz et al 2000). Finally, retinoids have been shown to modulate T cell responses and inhibit chemotactic responses and activation of polymorphonuclear leukocytes, such as neutrophils, eosinophils and basophils (Warren and Griffiths 2008).

Cyclosporine, a calcineurin inhibitor, is often used in the treatment of psoriasis. It binds ciclophilin (Ho et al 1996) and the resultant cyclosporine-ciclophilin complex inhibits the activity of calcineurin phosphatase, which is responsible for the dephosphorylation of nuclear activated T cells. This dephosphorylation stimulates the translocation of nuclear factor of activated T cells into the nucleus, thus activating T cells and stimulating the release of cytokines, such as IL2 and IFNγ.

So-called “biological” agents (monoclonal antibodies) are increasingly used in the treatment of refractory psoriasis. Development of biologics has arisen from our increased understanding of the involvement of the immune system in psoriasis (Rich and Bello-Quintero 2004). Biologics that have proved most successful in the treatment of psoriasis are those that target TNF signalling. Examples of anti-TNF biologicals are infliximab and etanercept (Krueger and Bowcock 2005).

Phototherapy is an important systemic therapy in the treatment of psoriasis. It has long been known that sunlight is beneficial to those with psoriasis and is often used in conjunction with other topical or systemic therapies (Lebwohl and Ali 2001). PUVA, which involves ingestion of 8-methoxypsoralen (8-MOP) followed by exposure of the skin with UVA has proven to be very effective, however its use is declining due to the association with the development of cutaneous malignancies (Lebwohl and Ali 2001). To lower the risk of malignancies developing, PUVA is now used in association with other treatments for psoriasis and in some cases, is alternated with other therapies.

1.3 Animal Models of Psoriasis

Numerous animal models targeting various pathological mechanisms and exhibiting certain aspects of psoriasis have been generated. For example, animal models that disrupt various components of the immune system have been developed to examine their role in psoriasis disease development. For example, the IκB subunit IKKB, that has a role in the negative regulation of NFκB signalling, has been specifically deleted in the epidermis, resulting in a severe inflammatory skin disease, dependent on the dermal expression of TNFα (Pasparakis et al 2002). These mice show altered differentiation and thickening of the epidermis, but also several other characteristics that are not

representative of psoriasis, such as keratinocyte apoptosis and T cell-independent inflammation. Deletion of IKKA, another catalytic subunit of IκB, results in epidermal abnormalities without inflammation, therefore is not representative of psoriasis (Gudjonsson et al 2007). Despite replicating some aspects of psoriasis, there are characteristics of this model that do not appear in psoriasis, thus making this model unsuitable for studying the pathology of psoriasis.

Transgenic mouse models that mimic the overexpression of cytokines thought to be crucial in psoriasis also fail to replicate many aspects of the disease. Thus, overexpression of TNFα targeted to the epidermis caused retardation of hair growth, inhibition of adipose production, signs of fibrosis and immune infiltration in the dermis with subsequent necrosis and cachexia (Cheng et al 1992). While this data suggests that TNFα expression by keratinocytes plays an important role in modulating the immune response it doesn't clarify how TNFα is involved in psoriasis. Another important cytokine in psoriasis, IFNγ, induces keratinocyte proliferation when injected into normal skin (Barker et al 1993). Mice that express IFNγ in the suprabasal layer of the epidermis display reddened skin, growth retardation, hair loss and flaky skin lesions. In addition, keratinocyte proliferation was increased and there was marked epidermal thickening, parakeratosis and an inflammatory infiltrate was also observed (Carroll et al 1997). However these mice are more representative of atopic dermatitis than psoriasis. Similarly, mice that constitutively express the p40 subunit of IL12 and IL23 in basal keratinocytes develop an eczematous phenotype, which can be replicated by subcutaneous injections of IL-12 (Kopp et al 2001). This is disappointing given the body of evidence implicating IL-23/IL-12 signalling in the pathogenesis of psoriasis.

Due to the polygenic nature of psoriasis, the development of an animal model that replicates all aspects of the disease is unlikely. In light of these limitations, xenograft models, where the skin of psoriasis patients is transplanted onto immunosuppressive mice have been generated. Three different strains of immunocompromised mice have been utilised, nude mice, severe combined immunodeficiency (SCID) mice and AGR129 mice. The most promising of the three strains is the AGR129 mouse. AGR129 mice are deficient in type I and type II IFN receptors and also lack the recombinase activating gene-2 gene (Boyman et al 2004). In addition, these mice also lack T- and B-cells and have immature NK cells that display impaired cytotoxicity activity *in vitro* and *in vivo*. Boyman et al showed that transplantation of non-involved skin from a psoriatic patient spontaneously develops into a psoriatic skin lesion (Boyman et al 2004). Following the transplantation of the skin onto the AGR129 mouse, T cells present in the graft undergo local proliferation and activation, a process that is inhibited by treatment of the grafts with anti-CD3 antibody (Boyman et al 2004, Gudjonsson et al 2007). The expansion of $CD8^+$ T cells is particularly important for the development of the lesion in the graft.

In SCID mice, injecting superantigen-stimulated blood-derived immunocytes under the transplanted skin was able to induce the development of a psoriatic lesion (Wrone-Smith and Nickoloff 1996). SCID and AGR129 xenograft models have provided evidence supporting the belief that psoriasis is a T-cell-mediated disease. Despite their ability to mimic psoriasis, all xenograft models are limited by their low throughput nature, the need to obtain human skin for each mouse, and the immune-deficient background for their generation, which inherently deviates from the highly immuno-active context of psoriasis lesions. None of these models have successfully been used in a high throughput fashion for testing of novel treatments to date. Nonetheless, these

xenograft models are currently the best available for the replication of the human disease.

Some of the most interesting models that have been developed target growth factors and signal-transducing molecules, such as STAT3 and VEGF. STAT3 is known to have a role in cell proliferation, survival and cell migration, making it an interesting candidate for psoriasis disease progression. STAT3 has also been shown to play a role in wound healing (Sano et al 1999) and is activated in psoriasis (Sano et al 2005). To this end, Sano and colleagues developed a constitutively active STAT3-expressing mouse model that develops lesions that are similar to psoriasis either spontaneously or in response to tape stripping (Sano et al 2005). It mimics many of the key histological features displayed in psoriasis, such as acanthosis, loss of the granular layer and parakeratosis. In addition, dilated blood vessels and a leukocyte infiltrate were also observed. This infiltrate is composed mainly of $CD4^+$ T cells in the upper dermis and epidermis, with few $CD8^+$ T cells localised in the epidermis. Interestingly, grafts from these mice transplanted onto immunodeficient mice showed no phenotype, however following injections of T cells from the STAT3 mutants under the graft, a psoriatic phenotype was observed. Therefore development of lesions in this model is clearly dependent on T cell activation (Gudjonsson et al 2007, Sano et al 2005). A potent activator of STAT3, IL-20, has been expressed by targeting its expression to the basal layer. This targeted expression results in abnormal keratinocyte differentiation with expression of the regenerative maturation marker keratin 6 (K6) (Blumberg et al 2001). A thickened hyperkeratotic epidermis and compact stratum corneum were also observed. Supporting the role of STAT3 in psoriasis, a mouse model in which VEGF was specifically expressed in the basal layer was established. VEGF signals via STAT3 and is able to induce proliferation of human keratinocytes (Gudjonsson et al 2007). Mice that

overexpress VEGF in the basal layer develop a psoriasis like phenotype and also display a similar response to injury as seen in the Koebner phenomenon (Xia et al 2003). These mice showed hyperplastic dermal blood vessels, epidermal thickening with dysregulated keratinocyte differentiation and characteristic inflammatory infiltrates. Furthermore, the phenotype is reversed by treating mice with a VEGF antagonist (Xia et al 2003).

Although many of these models do not exhibit a "pure" psoriasis phenotype, they do provide insight into the possible roles of various dysregulated components of disease pathogenesis. However, many of the models discussed here have not been tested to assess current psoriasis treatment efficacy, a parameter that could, if efficacy were shown, support the legitimacy of the model as a representation of human psoriasis. Thus, there remains a need for a good model that replicates many, if not all, aspects of the disease.

1.4 PPARδ

1.4.1 Family of Peroxisome Proliferator Activated Receptors (PPARs)

Peroxisome proliferator activated receptors (PPAR) are ligand activated transcription factors that belong to the nuclear hormone superfamily. There are 3 different isotypes of PPARs, PPARα (NRIC1), PPARβ/δ (NRIC2, referred to as PPARδ) and PPARγ (NRCI3) (Michalik et al 2006). PPARγ exists as two splice variants, PPARγ1 and PPARγ2, which arose during evolution by gene duplication that gave rise to PPARγ. For the PPARs to be able to regulate transcription, unlike other nuclear hormone receptors, which form homodimers following activation, PPARs require heterodimerisation with the retinoid X receptor (RXR), a member of the same

superfamily of receptors (Michalik et al 2006). These PPAR/RXR heterodimers can form in the absence of a ligand and, when activated are able to mediate transcription by binding to a specific DNA sequence element, the peroxisome proliferator response element (PPRE) on the target gene (Michalik et al 2006). The PPRE is composed of 2 half-sites, which occur as a direct repetition (DR1 repeats) of the consensus sequence AGGTCA, with a single nucleotide spacing in between the repeats. These PPRE sequences are present in one or multiple copies in the promoter region of target genes. The PPAR/RXR heterodimer bind to the 5' and 3' half-sites of PPRE respectively, with the 5' flanking region determining the selectivity of binding of different PPAR isotypes.

1.4.2 Structure of PPARs

All 3 PPAR isotypes are similar in structure, differing only in the percentage of homology between the DNA binding domain and ligand-binding domain. PPARs have an N terminal domain, termed the A/B domain, which contains a ligand-independent transcriptional function and which in PPARα and PPARγ can be regulated by MAPK phosphorylation (Hihi et al 2002). The C domain forms the DNA binding domain, which is characterised by 2 zinc-finger-like motifs, similar to those that have been described for steroid receptors. The E/F domain contains the ligand-binding domain, that contains a ligand-dependent transactivation function, AF-2, which is able to interact with transcriptional co-activators, regulating the transcriptional control of the PPAR/RXR heterodimer (Michalik et al 2006). The ligand-binding domain is depicted in the 3D crystal structure filled with white to mark the location on each PPAR isoform (figure 1.5).

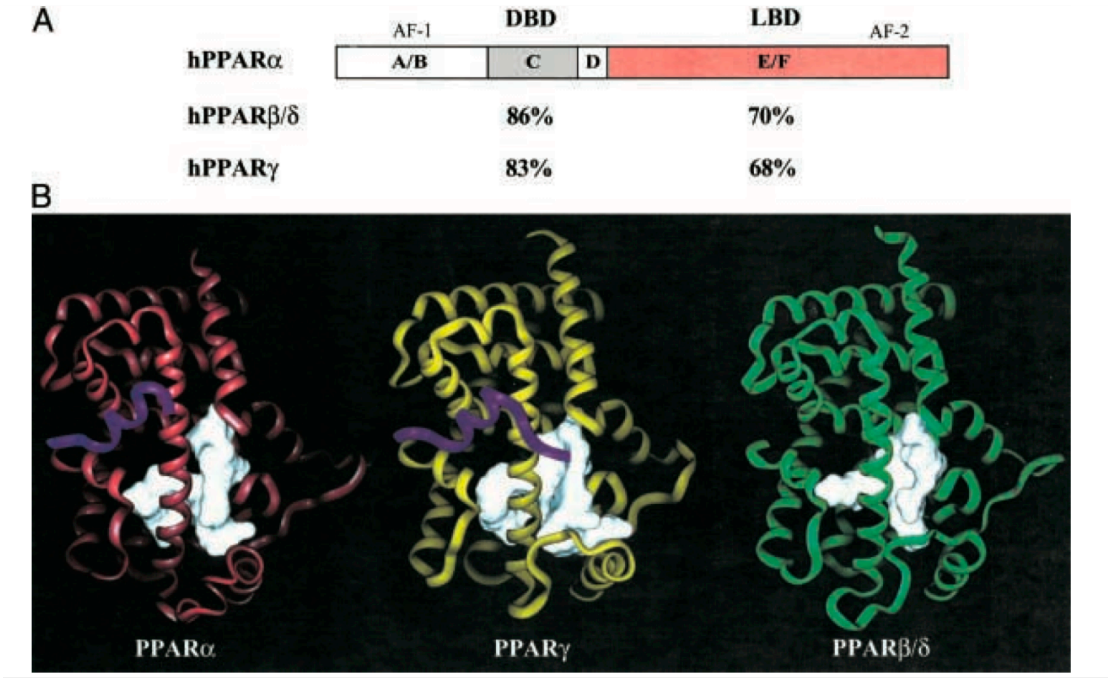

Figure 1.5: Structure Of PPARs*. A shows a schematic representation of PPAR, depicting the functional domains, which are described in the text. B shows the 3D structure of the ligand-binding domain of each of the PPARs. Modified from (Hihi et al 2002)*

Binding of ligands to the ligand-binding site induces a conformational change that favours the dissociation of co-repressors and stimulates the heterodimerisation with RXR and the recruitment of co-activators, which in turn recruits RNA polymerase and initiates remodelling of histones near regions of DNA containing DR1 motifs, thereby increasing the transcription of target genes (Peters and Gonzalez 2009).

1.4.3 PPARδ Function

Unlike PPARα and PPARγ, which are only expressed in specific tissues, PPARδ expression is found in virtually all tissues, in varying levels (Peters and Gonzalez 2009). Expression levels appear to be dependent on extent of cell proliferation and

differentiation that occurs in a particular tissue (Michalik et al 2006), with expression highest in skin, gut, placenta, skeletal muscle, adipose tissue and brain. PPARδ has a role in cell proliferation, cell differentiation, fatty acid catabolism and development. The development of specific PPARδ synthetic agonists such as GW501516 (Sznaidman et al 2003) has contributed towards elucidating its role in lipid metabolism. Dosing of insulin-resistant obese rhesus monkeys with the specific PPARδ agonist GW501516 for 4 weeks resulted in the normalising of fasting glucose and insulin levels, increased high-density lipoprotein cholesterol and a reduction in low-density lipoprotein cholesterol (Luquet et al 2004, Oliver et al 2001). Increased insulin sensitivity and a decrease in adiposity in obese mice when treated with the same compound, confirms that PPARδ plays a role in fatty acid metabolism. These data support the possible development of PPARδ as a target for anti-obesity and type 2 diabetes treatments. In addition to its role in fatty acid catabolism, PPARδ has been shown to play a role in lipid metabolism, particularly in skeletal muscle. This fits with PPARδ being the predominant isoform expressed in skeletal muscle (Holst et al 2003).

Further to the actions of PPARδ on fatty acid catabolism and lipid metabolism, there has been some debate about its role in proliferation, particularly in keratinocytes, where it is highly expressed. The actions of PPARδ on proliferation appear to be dependent on the cellular context in which it is expressed (Romanowska et al 2008). Exacerbation of hyperplasia by phorbol ester in PPARδ-deficient mice suggests an inhibitory role in keratinocyte proliferation (Peters et al 2000). Kim et al also reports inhibition of keratinocyte proliferation by PPARδ (Kim et al 2006), with evidence from the null PPARδ transgenic mouse. Additionally, in a subsequent study by members of the same group, inhibition of proliferation in N-TERT cells, an immortalized keratinocyte cell line, was also shown (Burdick et al 2007). In contrast to this data, Romanowska and

colleagues demonstrated that PPARδ enhances proliferation in primary human keratinocytes following activation with a selective ligand (Romanowska et al 2008). Critics argue that the concentrations of the specific agonists used results in activation of the two other PPAR isoforms, which have known stimulatory effects on proliferation (Peters and Gonzalez 2009). The precise role of PPARδ in keratinocyte proliferation is currently still debated.

1.4.4 The Role of PPARδ in Psoriasis

PPARδ is highly expressed in lesional skin of psoriatic patients. There is strong evidence to suggest a protective anti-apoptotic role of PPARδ in wound healing. In a study by Di Poi and colleagues, keratinocytes from PPARδ-null mice were more sensitive to growth factor withdrawal and anoikis (suspension-induced apoptosis) than +/+ PPARδ primary keratinocytes (Di-Poi et al 2003). In agreement with these results, when HaCaT cells are stimulated with a PPARδ agonist, L-165041, they are more resistant to apoptosis induced by growth factor withdrawal or anoikis. The expression of PPARδ is also increased following a wound, particularly at the wound edge (Sertznig et al 2008). These authors showed that this occurs in response to inflammatory cytokines, such as TNFα and IFNγ via an AP1-dependent stress response pathway, which is activated during wound healing. It is presumed that PPARδ expression at the wound edge would promote keratinocyte survival through this anti-apoptotic function, facilitating wound closure.

As psoriasis has been described as an exaggerated wound response, it is not surprising that PPARδ is overexpressed in psoriatic lesions (al Yacoub et al 2008, Westergaard et al 2003). Expression arrays and immunohistochemical data from lesional psoriatic skin

confirmed that PPARδ is overexpressed in psoriasis (Romanowska et al 2008, Westergaard et al 2003). In addition, PPARδ was shown to interact with NFκB signalling, which was implicated in the pathogenesis of psoriasis (Westergaard et al 2003). In contrast to this, PPARδ has been described to be capable of inhibiting NFκB nuclear translocation induced in response to inflammatory cytokines, thus allowing PPARδ to exert anti-inflammatory effects (Barroso et al 2011, Peters and Gonzalez 2009). In the study conducted by Romanowska and colleagues, it was shown that PPARδ induces Heparin-Binding EGF-Like Growth Factor (HB-EGF) (Romanowska et al 2008). HB-EGF is of interest due to being upregulated in psoriasis and has been shown to induce epidermal hyperplasia, a key hallmark of psoriatic disease. This suggests that activation of PPARδ and subsequent activation of HB-EGF contribute to the hyperproliferative phenotype observed in the disease. In a different study by the same group, PPARδ was shown to be expressed in T cells from peripheral blood and T cells isolated from psoriatic tissue (al Yacoub et al 2008). PPARδ was shown to contribute to T cell proliferation and evasion of apoptosis, suggesting a role in the sustainment of T cell populations in psoriasis. The authors postulate that PPARδ is a type I IFN target and is responsible for mediating the observed pro-survival effects (al Yacoub et al 2008). Given these data, it could be that PPARδ is overexpressed in psoriasis, and may contribute to the maintenance of the disease via induction of HB-EGF and its anti-apoptotic effects exerted in T cell populations. It is therefore of interest to investigate whether overexpression or a constitutively active form of PPARδ *in vivo* may yield a hyperproliferative phenotype similar to psoriasis.

To date, there is little data to support an interaction between PPARδ and STAT3. However, PPARδ is capable of activating a variety of tyrosine kinases, such as TGF-α and HB-EGF (Romanowska et al 2008), which are instrumental in the activation of

STAT3. It is possible that either of these two kinases, which are upregulated in psoriasis, could contribute to the activation of STAT3. On the histological level of psoriasis, it appears that STAT3 and PPARδ have the same localisation in the epidermis (Sano et al 2005, Westergaard et al 2003). This would suggest that there is some form of interaction between these two molecules, albeit indirectly. In addition, it was recently shown that PPARδ is capable of binding to a STAT3 response element and enhancing STAT3 activity (Wu et al 2010). The limited data available support the hypothesis that PPARδ and STAT3 interact; therefore one can assume that since both are overexpressed in psoriasis, they both may contribute to disease progression and sustainment.

1.5 STAT3

1.5.1 STAT Family of Transcription Factors

Signal Transducers and Activators of Transcription (STAT) are a family of signal transducers involved in the regulation of gene transcription and mediating the effects of cytokine signalling. There are 7 family members described to date, STAT1, 2, 3, 4, 5a, 5b and 6. STATs were discovered while investigating the downstream regulation of IFN signalling (Horvath 2000). These different family members are highly conserved and have crucial roles in the regulation of cellular functions in response to extracellular cytokine and growth factor signals. All STATs signal in roughly the same manner, following activation of a receptor with intrinsic tyrosine kinase activity, STATs bind to the phosphorylated tyrosine residues via a src homology 2 (SH2) phosphotyrosine-binding domain. They are then phosphorylated at either tyrosine or serine residues and this allows them to form homodimers and subsequent translocation to the nucleus to regulate gene transcription (Horvath 2000). However, some isoforms have the ability to

form heterodimers with other members of the STAT family, for example STAT2 is able to form a heterodimer with STAT1, which functions downstream of IFNα, STAT1 and STAT3 downstream of IL6 and STAT5a and STAT5b downstream of growth factors, such as EGF. The majority of the STAT homodimers bind and recognise variants of GAS-like DNA elements $TTN_{5-6}AA$, showing preference for specific sequence variations of the internal nucleotides of the inverted repeat (Horvath 2000).

1.5.2 STAT Structure

The structure of STATs is virtually the same in all isoforms, differing only in the transcriptional activation domain (TAD) (figure 1.6). The N domain, located at the N-terminus of STATs has proven important for stablising interactions between dimers bound to promoters (John et al 1999). The coiled-coil region forms part of the core fragment of the STATs, its exposed surfaces present many binding sites for protein interactions (Horvath 2000). The coiled-coil region is linked to the DNA-binding domain, which binds specific sequences in the promoters of target genes. The linker domain composed primarily of α-helices separates the DNA-binding domain from the phosphotyrosine-binding SH2 domain and has also been implicated in transcription. The SH2 domain is involved in the binding to phosphorylated tyrosine residues on the activated receptors. Finally the tyrosine residue is followed by amino acids that are C-terminally located that form the transcriptional activation domain (TAD). TAD is essential for STAT protein functions (Horvath 2000).

Figure 1.6: Schematic Showing General STAT Structure. *ND represent the N-domain involved in dimer interactions (yellow), Coil coil domain is responsible for protein-protein interactions (green), DNA is the DNA binding domain (red), LD is the linker domain involved in transcription (orange), SH2 is the SH2 domain involved in receptor binding and dimerisation (blue). All STATs have a tyrosine residue (Y) that is phosphorylated upon receptor binding causing them to become activated, many of the STATs also have serine residue in their transcriptional activation domain (purple). Modified from (Horvath 2000).*

1.5.3 STAT3 Function

STAT3 is implicated in many cellular processes. Knockout of STAT3 results in embryonic lethality, pointing towards a role for STAT3 in development, however the mechanism for the activation of STAT3 in this particular process is still unclear (Takeda and Akira 2000). T-cells deficient in STAT3 show impaired IL6-induced cell proliferation, due to a lack of IL-mediated prevention of apoptosis of these cells (Takeda et al 1998). This suggests that STAT3-mediated signalling is responsible for the anti-apoptotic signal and leads to enhanced T cell proliferation. Mice with the deletion of STAT3 in these cells are more susceptible to endotoxin shock, with the serum concentration of inflammatory cytokines being increased (Takeda et al 1999). IL-10, which normally has an inhibitory effect on the production of cytokines by macrophages and neutrophils, no longer demonstrates an effect in STAT3 deficient cells (Takeda et al 1999). In addition, these mice show an elevated Th1 response and develop

chronic enterocolitis. STAT3 is evidently involved in the mediation of cytokine production by macrophages and neutrophils, which appears to be dependent on IL10. Like the STAT5 isoforms, STAT3 also plays a role in mammary gland development (Chapman et al 1999).

1.5.3 The Role of STAT3 in Psoriasis

Several *in vitro* and *in vivo* studies support the role of STAT3 in psoriasis (Sano et al 1999, Sano et al 2005). STAT3 has been strongly implicated in wound healing. Mice where STAT3 is specifically deleted from the epidermis, have retarded wound healing (Sano et al 1999). However, there is no difference in the dermal responses to wounds such as granulation, inflammation and the formation of new blood vessels in these STAT3 deficient mice. This directly suggests that STAT3 is primarily involved in epidermal regeneration and not in the development of dermal components.

In addition to the strong evidence suggesting a role for STAT3 in keratinocyte biology, immunohistochemical analysis of psoriatic skin revealed a strong upregulation of STAT3 throughout the epidermis (Sano et al 2005). In the same study, Sano and colleagues demonstrated that keratinocyte specific overexpression of STAT3 results in a mouse model that develops psoriatic-like lesions, as described in section 1.4. Perhaps the strongest evidence for a role of STAT3 in psoriasis is the observation that application of a JAK2 inhibitor directly to the skin effectively heals psoriasis plaques in humans. Thus, Duffin and colleagues recently described the use of a JAK1/JAK2 small molecule inhibitor, INCB018424, which was tested in two clinical trials. This inhibitor was able to inhibit acanthosis and production of IL-22 induced by intra-dermal IL-23 in the murine model and in patients reduced transcriptional changes consistent with

decreases in Th1 and Th17 lymphocyte activation, decreased epidermal hyperplasia and dendritic cell activation (Duffin et al 2010). In addition, following a second double blind clinical trial, total lesion score of patients revealed a 2-fold decrease over vehicle control at day 84 (Duffin et al 2010). These data support a role for STAT3 signal transduction following phosphorylation by JAK in psoriasis disease progression.

1.6 Aim

Based on the data outlined above, we sought to develop a transgenic mouse model that overexpressed PPARδ. This thesis details the characterisation and development of a model overexpressing PPARδ under the control of the CYP1A1 enhancer. To address the effectiveness of this model, the following were examined, 1) any phenotypic changes that occur following induction of the transgene with a specific PPARδ ligand 2) the localisation of PPARδ in induced and non-induced PPARδ transgenic mice, 3) whether there is any immune system activation in induced mice, similar to that of the human disease. In addition, STAT3 is highly overexpressed in psoriasis, as discussed previously. To investigate the contribution of STAT3 to the development of psoriasis-like disease in PPARδ transgenic mice, the following were examined, 1) to investigate the expression and activation levels of STAT3 in a psoriasis-like in the vivo model, 2) to ascertain the effect of STAT3 inhibition on disease development in the same model and finally 3) to determine the effects of STAT3 on interferon response genes deemed important in psoriasis pathogenesis.

Chapter 2: Materials and Methods

2. Materials And Methods

2.1 Chemicals, Reagents And Buffers

All general chemicals were of molecular biology grade and obtained from Sigma-Aldrich (Gillingham, Dorset, UK), VWR International (Poole, Dorset, UK), Fisher Scientific (Loughborough, Leicestershire, UK) unless otherwise stated.

2.2 Ethics Statement

All work involving animals was approved by the Tayside Ethics Committee. Storage and use of all tissues included in the work presented here was approved by the Tayside Committee on Medical Research Ethics B (REC ref. Nr. 07/S1402/90).

2.3 Home Office Licence

In order to carry out experiments involving animals, a personal licence from the Home Office must be obtained. This was awarded in June 2009 following training and a written exam granting permission to the holder to carry out regulated procedures on living animals. This licence does not authorize the licensee to perform any of the procedures specified in it unless they are carried out in the course of a project for which there is a project licence in force under the Animals (Scientific Procedures) Act 1986. The project licence number used for this work is 60/3800 and the personal licence number used is PIL 60/12167.

2.4 PPARδ Immunohistochemistry – Human Skin

Human skin biopsies embedded in paraffin were obtained from Tayside Tissue Bank. Prior to biopsy, patients gave written consent to storage and analysis of biopsy samples. Paraffin sections of skin were prepared by Tayside Tissue Bank. Following preparation of sections, they were deparaffinised by heating for 1 hour at 60°C then subjected to a series of alcohol washes in the following order,

Histoclear – 5 minutes

Histoclear – 5 minutes

Absolute Alcohol (100% IMS) – 3 minutes

Absolute Alcohol (100% IMS) – 3 minutes

Methylated Alcohol (95% IMS) – 3 minutes

Following deparaffinisation, sections were subjected to antigen retrieval to unmask antigens that may have become masked during tissue fixation. To achieve this, sections were boiled in 10mM citric acid, pH=6.0 for 10 minutes. To remove residual citric acid, sections were washed with dH_2O for a total of 3 times. Sections were incubated with hydrogen peroxide (3%) for 10 minutes to reduce endogenous peroxidases. Sections were washed in between incubation steps with TBST. Using VECTASTAIN® Universal Elite® ABC Kit (Vector Labs, Cat.no.: PK-6200), sections were incubated with normal blocking serum (5ml TBST + 1 drop Normal Blocking Serum Stock) for 20 minutes. Excess serum was blotted from each section before proceeding with incubation with primary antibody against PPARδ. Primary antibody was diluted in normal blocking serum, 1:100 and applied to sections, which were then incubated overnight at 4°C.

Antibody	**Manufacturer**
Monoclonal Mouse Anti-human PPARδ/NR1C2, Clone: K9436	R&D Systems, Cat.no.: PP-K9436-00

Table 1: Antibodies Used In PPARδ Immunohistochemistry. *The same antibody was used for both human and mouse PPARδ immunohistochemistry.*

Following primary antibody incubation, sections were incubated with diluted biotinylated secondary antibody (5ml TBST + 2 drops Normal Blocking Serum Stock + 2 drops of Biotinylated Antibody Stock), for 30 minutes at room temperature followed by a 30 minute incubation with VECTASTAIN® Elite® ABC Reagent (5ml TBST + 2 drops Reagent A + 2 drops Reagent B). To visualise positive staining, a peroxidase substrate, DAB (Peroxidase Substrate Kit, Vector Labs, Cat.no.: SK-4100) was applied for 30 seconds, which was prepared as follows,

5ml of distilled H_2O

2 drops of Buffer Stock Solution

4 drops of DAB Stock Solution

2 drops of Hydrogen Peroxide Solution.

Sections were counterstained with Mayers Hematoxylin for 2 minutes followed by dehydration via a series of graded alcohol washes. Coverslips were mounted using DPX on a Leica Fully Automated Glass Coverslipper.

2.4 PPARδ Expression System

PPARδ transgenic mice were already available in this laboratory upon starting this work. Full length human PPARδ was cloned into mice downstream of a CYP1A1 promoter as follows. Plasmids encoding human PPARδ were prepared and the PPARδ coding sequence was amplified using primers PRMG15 (5'-CTAGTCTAGAATGGAGCAGCCACAGGAGGAAGC-3') and PRMG3 (5'-CTAGTCTAGATTAGTACATGTCCTTGTAGATCTCCTG-3'). PCR products were cleaved using *Xba*I and cloned into plasmid pUHD10-3 shown in Figure 2.1.

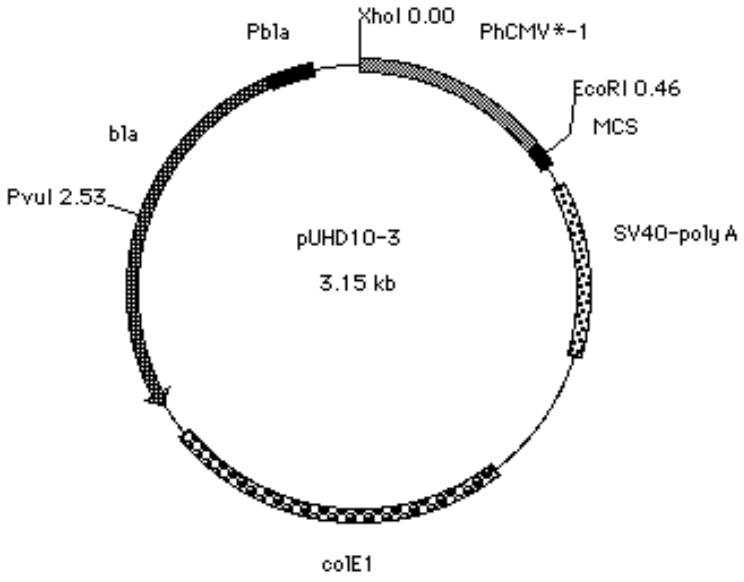

Figure 2.1: Plasmid Orientation Of pUHD10-3 Used For PPARδ Cloning.

Unpublished data from M. Gossen.

Image from http://www.zmbh.uni-heidelberg.de/bujard/reporter/pUHD10-3.html.

The resultant plasmid was pMGD7. Inserts were confirmed by sequencing, then cleaved out using *Bam*HI and ligated into plasmid pAHIRI-β-gal (shown in figure 2.2), which was then cleaved with *Bgl*II, thus removing the β-galactosidase reporter gene. This plasmid was designated pMGD72.

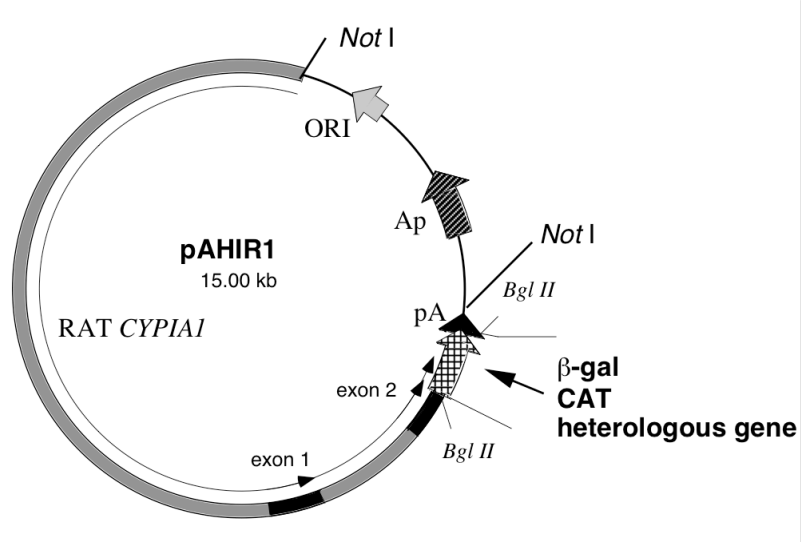

Figure 2.2: Plasmid Orientation Of pAHIRI-β-gal. *The plasmid contains a region of the rat CYP1A1, pA, polyadenylation site; Ori, origin of replication; and Ap, the ampicillin resistance gene. It also contains a β-galactosidase reporter gene (β-gal) to produce pAHIR1β-gal (Campbell et al 1996).*

The orientation of the inserts were confirmed by restriction endonuclease analysis and sequencing to ensure the inserts were ligated in the correct order. The plasmid pMGD72 was then microinjected into pro-nuclei of C57 Black 6 mice crossed with CBA F1 fertilised eggs.

2.5 DNA Extraction And PPARδ Genotyping

Ear tags were obtained from all mice in the line in preparation for genotyping and to assign a unique number to each mouse so that they were easily identifiable from littermates. Mice were tagged according to the designation shown in Figure 2.3.

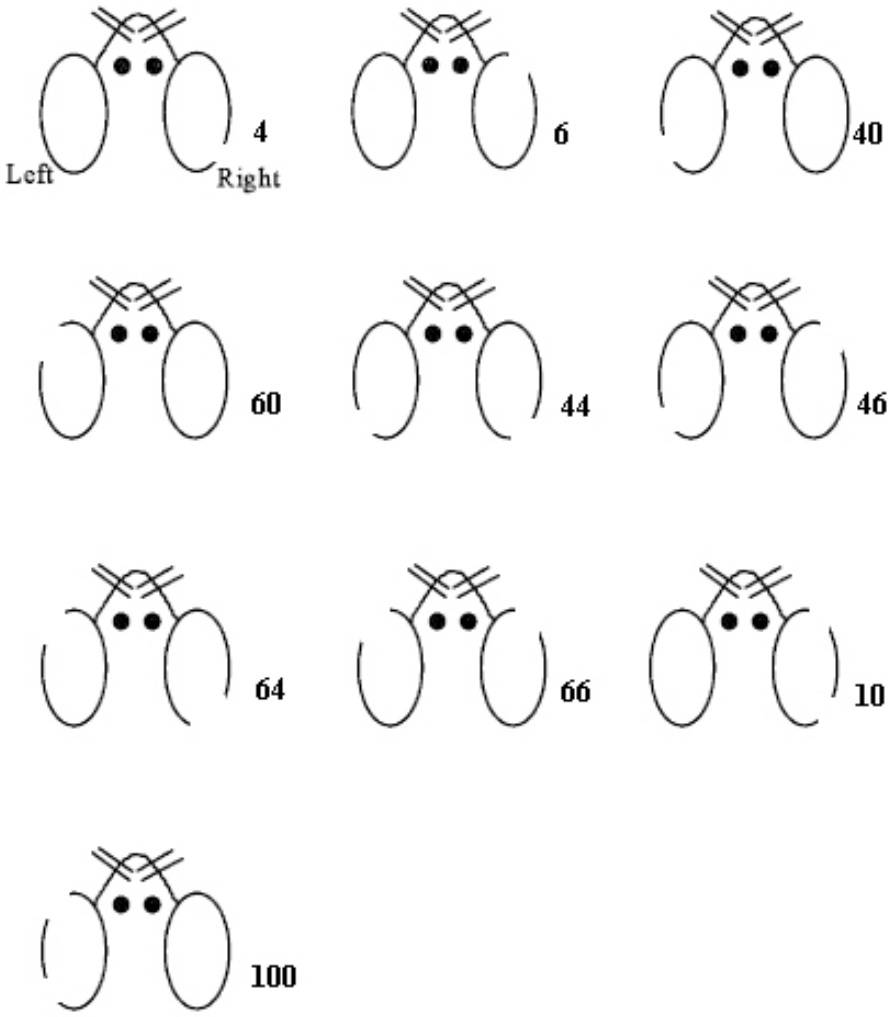

Figure 2.3: Ear Designations For Mouse Identification. *All mice in litters underwent ear tagging for genotyping and identification purposes. Shown above is the guide according to which all mice were ear tagged.*

Following collection of ear tags, DNA was extracted using the Macherey-Nagel Nucleospin Tissue kit. Ear tags were incubated overnight at 56°C with vortexing in an Eppendorf Thermomixer Comfort, suspended in 180µl T1 buffer with 25µl of Proteinase K solution added. Samples must be fully covered with buffer and tubes were flicked to ensure this. Samples were lysed with the addition of 200µl Buffer B3 and heated at 70°C for 10 minutes. DNA binding conditions were adjusted via the addition of 210µl of 96-100% ethanol. Samples were thoroughly mixed via aspiration with a pipette before being transferred to a NucleoSpin® Tissue Column. The column was then centrifuged for 1 minute at 11,000g and the flow-through discarded. The silica membrane was washed twice, once with Buffer BW followed by centrifugation, then with Buffer B5 followed by centrifugation, with the flow-through discarded each time. Centrifuging for 1 minute at 11,000g dried the membrane. Columns were transferred to a 1.5ml microcentrifuge tube and DNA was eluted by the addition of 40µl of prewarmed (70°C) Buffer BE. Samples were incubated at room temperature for 3 minutes then centrifuged for 1 minute at 11,000g. Eluted DNA was stored at -20°C until use. Resultant DNA Concentrations from the ear tags were determined using a NanoDrop 2000 Spectrophotometer. The amount of sample needed to give 20ng of DNA was then calculated and used in a PCR reaction. The way this was calculated is shown in table 2

Mouse number	DNA Concentration (ng/µl)	µl DNA(20ng)	µl H_2O (to 2.5µl)	DNA Total (ng)
n	x	=20/x	=2.5-(20/x)	=x*(20/x)

Table 2: Calculation To Determine Amount Of Sample Needed To Yield 20ng Of DNA For PCR. *DNA concentration used is an average of at least 2 similar values with a standard deviation of less than 1.*

Samples were pipetted into 0.2ml PCR tubes to give a total volume of 2.5µl. A master mix containing H_2O, 10X CoralLoad PCR Buffer, 5X Q-Solution, dNTP mix, primers mix and HotStarTaq Plus Polymerase was added to each sample. These reagents were from Qiagen (HotStarTaq DNA Polymerase, Cat.no.: 203203), selected for minimal optimization needed and that can deal with the large PCR product generated by the primers. The amounts of each reagent added was as follows,

For 1 sample,

13.8µl H_2O (to 25µl)

2.5µl 10X CoralLoad PCR Buffer

5µl 5X Q-solution

0.5µl dNTP (10µM each)

0.5µl Primers (356pRMG160 + 355pRMG159, 10µM each)

0.2µl HotStarTaq Plus Polymerase (=1U)

22.5µl master mix per sample

+ 2.5µl DNA

Primer Name	Sequence
355pRMG159-R	5'-CCAACCACCCTGTCCCAGCTTG-3'
356pRMG160-F	5'-ACAAACTCTGCCCTGCTCTATG-3'

Table 3: Primers Used In PPARδ Transgenic Mice Genotyping.

Samples were then run on a BioRad C1000 Thermal Cycler on the following program,

1: 95°C for 5 minutes

2. 94°C for 5 minutes

3. 60°C for 30 seconds

4. 72°C for 2 minutes

5. Go to 2 for 39 times

6. 72°C for 10 minutes

7. 4°C forever.

Products from the PCR were analysed using gel electrophoresis. Agarose gels were prepared by dissolving 2% (w/v) of Ultrapure® Agarose (Invitrogen, Cat.no.: 16500-500) in 1X TBE. Agarose was dissolved by heating in a microwave on high power until the powder was dissolved. Liquid agarose was then cooled to approx. 55°C, then SYBR® Safe DNA gel stain (Invitrogen, Cat.no.: S33102) was added, 10µl per 100ml. The liquid agarose was then poured into a gel cast, ensuring that no air bubbles form, before a comb was inserted and the gel allowed to set. PCR products were added to each well, 10µl per well. A 100bp DNA marker (Invitrogen, Cat.no.: 15628-019) was added in an adjacent lane to verify PCR product size. The gel was run at a constant of 120 milliampere for 40 minutes to 1 hour. Gels were analysed using UVIPRO Silver CCD camera with UV capability to visualize DNA bands on the gel.

2.6 PPARδ Immunohistochemistry – Mouse Tissue

Mice were fed on a 0.003% GW501516 powder diet for 2 weeks before being terminated in accordance with Schedule 1 killing procedures as set by the UK Home Office. Mice were killed either by cervical dislocation or in a CO_2 gas chamber. Organs were harvested by dissection, or in the case of the skin, shaved then dissected. All tissues were immediately stored in 10% formalin to preserve their structure and overall integrity. Samples were then given to Tayside Tissue Bank for paraffin embedding and sectioning. Sectioning, deparaffinisation, antigen retrieval and hydrogen peroxide protocols were the same as those used for human tissue.

Sections were washed with PBS in between incubation steps. Following washing, sections were incubated with a blocking solution (2.5ml PBS + 2 drops M.O.M Mouse Ig Blocking reagent stock solution) prepared from the M.O.M Immunodetection Kit – Peroxidase (Vector Labs, Cat.no.: PK-2200) for 1 hour at room and then incubated with diluent (7.5ml PBS + 600µl Protein Concentrate stock solution = M.O.M diluent) for 5 minutes at room temperature. Excess diluent was blotted from the slides and primary antibody was added (1:1000 in M.O.M diluent, see table 1 for antibody details) to each slide and then incubated for 30 minutes at room temperature. Secondary antibody (2.5ml M.O.M diluent + 10µl M.O.M Biotinylated Anti-Mouse IgG reagent stock solution) was added to the sections, which were incubated at room temperature for 10 minutes. Sections were incubated with ABC reagent as outlined in section 2.4.

2.7 Preparation Of GW501516 Diet For *In Vivo* Experiments

A concentrated stock of 0.3% GW501516 (w/w, custom – synthesised by AF-Pharmaceuticals, UK, to ≥98% purity) in powder diet was prepared. All subsequent diet was made by diluting the concentrated stock. All *in vivo* experiments were performed using 0.003% GW501516 diet unless otherwise stated. To make 500g of 0.003% diet, the following was carried out.

It was calculated how much 0.3% stock would be needed to give a final concentration of 0.003% in 500g. The amount of GW501516 in 0.3% stock is 0.3g in 100g, therefore for a 0.003% diet, it would be 0.003g in 100g, meaning 0.015g in 500g. Thus the amount of 0.3% stock needed to yield 0.015g is 5g. This amount of concentrated diet was weighed out and made up to 500g with normal RMI diet (supplied in house by Medical Resource Unit). This mix was transferred to a blender and mixed for 10 minutes to ensure homogeneous reconstitution. Once mixed, the diet was transferred to a container and stored at 4°C when not in use.

2.8 Recording Of Phenotype – Clinical Photographs

For the taking of photographs to document the phenotype observed upon systemic induction with GW501516 containing diet, mice were manually restrained by scruffing of the neck with the index and middle finger, followed by securement of the tail by additional fingers. Feet were restrained as needed by remaining free fingers. Mice were held against a plain background for photographing. In addition, before each mouse was photographed, the cage card for that particular cage was also photographed to identify mice in the experiment.

2.9 H&E Histology

Samples were collected as described in section 2.6 and prepared as described in section 2.4. The H&E histology was performed using a Leica Autostainer provided by Tayside Tissue Bank. Deparaffinisation was performed as described previously. Sections were washed with H_2O followed with staining with Harris' Haematoxylin. Wash 2 with H_2O. Sections were incubated in 0.1% acid alcohol for 1 minute followed by a 3rd wash with H_2O. Sections were then incubated in STWS for 1 minute followed by a 4th wash with H_2O. Sections were additionally stained with Shandon Eosin for 30 seconds followed by a 5th wash with H_2O. Sections were rehydrated and coverslips mounted as described in section 2.4

2.10 $CD4^+$, $CD8^+$, $CD11c^+$ And $CD31^+$ Immunohistochemistry

Tissues were harvested from mice after 2 weeks of systemic induction with 0.003% GW501516 diet. Following termination, skin on the abdomen of mice was shaved to remove any hair that may be still present. Biopsies of skin were taken and immediately embedded in O.C.T and snap frozen in liquid N_2. Samples were stored at -80°C until use.

Samples were orientated and embedded further onto a metal chuck that fits onto a cryostat. Samples were sectioned at 5 microns thick and mounted onto Superfrost Plus slides (VWR International Ltd, Cat.no.: 631-9483). Following mounting onto slides, sections were dried at room temperature overnight.

Sections were fixed in ice-cold acetone for 10 minutes then dried for 15 minutes at room temperature. Sections were washed following each incubation with PBS. Sections were then incubated with 0.3% H_2O_2 for 10 minutes at room temperature followed by blocking using 0.8% FCS in PBS for 30 minutes at room temperature. Following blocking, excess serum was blotted from sections before incubation with primary antibodies, detailed in Table 4. Each antibody was used at a 1:50 dilution in the blocking buffer and incubated for 1 hour at room temperature.

Antibody	**Manufacturer**
Purified Rat Anti-Mouse CD4 (Monoclonal)	BD Biosciences, Cat.no.: 550278
Purified Rat Anti-Mouse CD8a (Monoclonal)	BD Biosciences, Cat.no.: 550281
Purified Hamster Anti-Mouse CD11c (Monoclonal)	BD Biosciences, Cat.no.: 550283
Purified Rat Anti-Mouse CD31 (Monoclonal)	BD Biosciences, Cat.no.: 550274
Biotinylated Goat Anti-Rat Ig (Polyclonal)	BD Bioscience, Cat.no.: 551013
Biotinylated Anti-Hamster IgG (Polyclonal)	BD Bioscience, Cat.no.: 551012

Table 4: Antibodies Used For Immunological Immunohistochemical Analysis. *All antibodies were used on mouse tissue only.*

Following incubation of sections with primary antibodies, sections were incubated with secondary antibodies, diluted 1:50 in blocking buffer for 30 minutes at room temperature. Details of antibodies that were used are shown in Table 4. After secondary antibody incubation, sections were incubated with Streptavidin-HRP (contained in both Anti-rat Ig HRP Detection kit and Anti-hamster Ig HRP Detection kit, details in Table 4) for 30 minutes at room temperature. Positive staining was visualised as previously described for PPARδ immunohistochemistry.

2.11 Western Blotting

Samples were collected from mice as previously described. Abdominal skin samples were immediately snap frozen and stored at -80°C until use. Once frozen, samples were homogenised under liquid N_2. This involved transferring a skin sample to a 2ml round bottomed Eppendorf tube. Once transferred, samples were immediately put back into liquid N_2 to ensure sample is sufficiently frozen. Samples were dipped into liquid N_2 with the lid open to allow a small amount of liquid to enter the tube. Meanwhile a large metal pestal that was shaped for the bottom of the 2ml tubes was cooled using liquid N_2. Using the pre-cooled pestal, samples were ground to a fine powder, ensuring to keep dipping samples into the liquid N_2 to keep them frozen.

Following tissue homogenisation, each sample was weighed to determine the exact weight of tissue present, then 5µl per mg of tissue of B* buffer was added, which was developed and described by Klose et al (Klose 1999) (B* buffer contains 960µl of Buffer B/Chaps + 40µl of 25x Proteinase Inhibitor cocktail stock. Buffer B contains 100mM phosphate buffer, pH =7.4, 20% glycerol and 0.2M KCl. 900µl of this stock was taken and 73mg of CHAPS added and 31µl of H_2O). To the samples containing B* buffer, 1µl of highly concentrated DNase was added. 3 glass beads were then added and samples were sonicated 5 x 10 seconds on ice. Samples were then centrifuged at 20,000rpm (approx. 30,000g) for 5minutes at 4˚C. Supernatant was removed and stored in a fresh pre-chilled Eppendorf tube. Following protein extraction, the concentration of resultant protein was determined by using Qubit Protein Assay Kit (Invitrogen, Cat.no.: Q33211) or by BCA Protein Assay Kit (Pierce, Cat.no.: 23225).

For SDS-Page analysis, gels were cast using BioRad 1.0mm spacer plates (BioRad, Cat.no.: 1653311) with BioRad short plates (BioRad, Cat.no.: 1653308). Gels were cast according to the following composition, shown in table 5 and table 6. In between separating gel and stacking gel casting, isopropanol was added on top of the separating gel to ensure that samples produced an even, straight band.

	Separating Gel 30ml							
	5%	**7.5%**	**8%**	**10%**	**11%**	**12.5%**	**15%**	**18%**
H_2O	17ml	14.5ml	14ml	12ml	11ml	9.5ml	7ml	4ml
Separating Buffer (1.5M Tris-HCl, pH=8.8)	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml	7.5ml
30% Acrylamide 37.5:1 ratio	5ml	7.5ml	8ml	10ml	11ml	12.5ml	15ml	18ml
10% SDS	300µl	300µl	300µl	300µl	300µl	300µl	300µl	300µl
TEMED	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl
10% APS	200µl	200µl	200µl	200µl	200µl	200µl	200µl	200µl

Table 5: Composition Of Separating Gel For SDS-Page Gel Electrophoresis.

	Stacking Gel 4%		
	6ml	**12ml**	**24ml**
H_2O	3.5ml	7ml	14ml
Stacking Buffer (0.5M Tris-HCl, pH=6.8)	1.5ml	3ml	6ml
30% Acrylamide 37.5:1 ratio	800µl	1.6ml	3.2ml
10% SDS	60µl	120µl	240µl
TEMED	6µl	12µl	24µl
10% APS	120µl	240µl	480µl

Table 6: Composition Of Stacking Gel For SDS-Page Gel Electrophoresis.

Samples were prepared by pipetting 40µg of protein, adding H_2O to 22.5µl then adding 7.5µl of Laemmli Sample Buffer (BioRad, Cat.no.: 161-0737). Samples were heated at 99° for 5 minutes, then allowed to cool to room temperature before being added to the gel. Into the first two lanes of each gel, 5µl of Precision Plus Protein Kaliedoscope

Standard (BioRad, Cat.no.: 161-0375) and 5µl of MagicMark™ XP Western Protein Standard (Invitrogen, Cat.no.: LC5602) were added for comparison of size of resultant bands. Gels were run for 1-2 hours at constant 0.02A, 40W.

Following SDS-Page to separate the proteins into their respective sizes, proteins in the gel were blotted onto Nitrocellulose membrane. The following set-up was used, 6 x Whatman papers, Nitrocellulose membrane, SDS-Page gel, 6 x Whatman paper. Prior to the gel being lain on top of the nitrocellulose membrane, the Whatman papers and membrane were pre-soaked in 1X Blotting Buffer (25mM TRIS-HCl 150mM Glycine 10% (v/v) Methanol) for 5 minutes. The membrane had additional 1X Blotting buffer added once orientated to prevent drying out before the gel was laid on top. This western blot sandwich was then placed in a BioRad semi-dry blotter and ran at constant 0.2A, 20W, 30V for 1 hour. Following blotting, membranes were incubated with Ponceau S stain for 5 minutes to ensure transfer had been successful. Images of Ponceau S stained membranes were recorded using an HP Scanner.

Membranes were washed with TBST following each incubation. They were then blocked for 30 minutes at room temperature with gentle shaking in 4% milk/TBST solution. Following blocking, membranes were incubated overnight at 4°C with gentle shaking with primary antibody diluted 1:1000 in 4% milk/TBST solution. Details of antibodies used in western blot analysis are shown in table 7.

Antibody	**Manufacturer**
Phospho-Stat3 (Tyr705) (D3A7) Rabbit Monoclonal Antibody	Cell Signalling, Cat.no.: 9145S
Stat3 Polyclonal Antibody	Cell Signalling, Cat. No.: 9132
Monoclonal Anti-GAPDH antibody produced in mouse, clone GAPDH-71.1,	Sigma, Cat.no.: G8795
ECL anti rabbit IgG, HRP conjugated (Polyclonal)	GE Healthcare, Cat.no.: NA934
Sheep polyclonal Secondary Antibody to Mouse IgG - H&L (HRP)	Abcam, Cat.no.: ab6808

Table 7**: **Antibodies Used In Western Blotting Analysis.

After incubation with primary antibodies, membranes were incubated with secondary antibody at a dilution of 1:2000 in 4% milk/TBST for 30 minutes at room temperature with gentle shaking. Excess TBST was removed from the membrane before being placed on a petri dish and ECL Plus (Amersham ECL PlusTM Western Blotting Detection Reagents, GE Healthcare, Cat.no.: RPN2132) was added and incubated in the dark for 5 minutes at room temperature. Excess ECL Plus was removed from the blot, which was then placed in a plastic sleeve before being exposed with a CCD camera with chemiluminescence capability.

After chemiluminescence for STAT3 and phospho-STAT3, membranes were washed three times with TBST for 5 minutes each after exposure then incubated with 1:1000 dilution of GAPDH in 4% milk/TBST for 1 hour at room temperature with gentle shaking. Membranes were then incubated with 1:2000 dilution in 4% milk/TBST of

secondary antibody for 10 minutes at room temperature with gentle shaking. Membranes were exposed and recorded as described in section 2.11.

2.12 Phospho-STAT3 Immunofluorescence

Frozen sections were prepared as previously described for immunological stainings using a cyrostat. Sections were incubated in ice-cold methanol for 15 minutes at -20°C. Following methanol fixation, slides were dried at room temperature for 15 minutes. Sections were washed with TBS following each incubation. To minimise the amount of antibody used, a waterproof barrier was drawn around the section on the slide using a Liquid Blocker Super Pap Pen. Sections were then incubated for 1 hour with blocking buffer (10% goat serum, 1% BSA, 0.3M Glycine) to reduce any non-specific background staining. Excess blocking buffer was blotted from sections which were then incubated with 1:50 dilution of Phospho-Stat3 antibody or 1:50 Phospho-Stat3 antibody + 1:100 Phospho-STAT3 Blocking Peptide in 1% BSA/TBS for 1 hour at room temperature. Following primary antibody incubation, sections were then incubated with secondary antibody, diluted 1:2000 in 1% BSA/TBS for 10 minutes at room temperature. See table 8 for antibody and blocking peptide details.

Antibody	Manufacturer
Phospho-Stat3 (Tyr705) (D3A7) Rabbit Monoclonal Antibody	Cell Signalling, Cat.no.: 9145S
Phospho-Stat3 (Tyr705) Blocking Peptide	Cell Signalling, Cat.no.: 1195
Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) (Polyclonal)	Invitrogen, Cat.no.: A-21202

Table 8: Antibodies Used In Immunofluorescence.

Following secondary antibody incubation, a small amount of ProLong® Gold antifade reagent with DAPI (Invitrogen, Cat.no.: P-36931) was added to the coverslip before being applied to the section. Sections were dried overnight in the dark before being sealed with clear nail polish and visualised using a fluorescent microscope which utilises oil to aid the focusing of the objectives.

2.13 WP1066 Treatment

10mg of WP1066 was dissolved into a solution containing DMSO and PEG300 in a 20 to 80 parts ratio to give a final concentration of 1.25µg/µl. This was achieved by centrifuging the WP1066 containing vial to ensure the entire drug was at the bottom. DMSO/PEG300 (20/80) was then added to the vial, which was sealed and vortexed until the entire drug had dissolved. Mice were administered 75µl of 1.25µg/µl solution of WP1066 or 75µl of DMSO/PEG300 (20/80) via intraperitoneal injection as described by Kong et al (Kong et al 2008). This was done three times weekly, in the morning to enable mice to be monitored throughout the day in case of any adverse effects. While

receiving injections of WP1066, mice were also being systemically induced with GW501516. H&E histology was performed as previously described.

2.14 TaqMAN Gene Expression Analysis

Sample sections were collected and processed as described in section 2.11. The following protocol was followed from Machery-Nagel RNA II Total RNA Isolation kit (Fisher Scientific, Cat.no.: NZ740955250). Lysis buffer, containing RA1 buffer and β-mercaptoethanol, was added to the samples, which were then vortexed vigorously. The lysate was filtered via centrifugation then RNA binding conditions adjusted through the addition of 70% ethanol. Samples were then loaded on to a new column and filtered via centrifugation. This ensured that RNA was then bound to the column membrane. The membrane was then desalted via addition of Membrane Desalting Buffer to maximise the efficiency of the DNase treatment. Samples were then incubated with an RNase free DNase for 15 minutes at room temperature, followed by a series of washes and drying of the membrane. RNA was eluted with RNase-free H_2O then snap-frozen and stored at -80°C.

RNA concentration was determined using the Qubit RNA Assay Kit (Invitrogen, Cat.no.: Q32855). Approximately 200-400ng of RNA was used to synthesis cDNA using a cDNA synthesis kit (SuperScript® VILO™ cDNA Synthesis Kit, Invitrogen, Cat.no.: 11754-050). For a single reaction, the following was done,

5X VILO™ Reaction Mix	4µl
10X SuperScript® Enzyme Mix	2µl
RNA	2.5µg
DEPC-treated water	to 20µl.

Once samples had been prepared for the reaction, they were put on the following program on a BioRad C1000 Thermo Cycler,

25°C for 10 minutes

42°C for 60 minutes

85°C for 5 minutes.

Approximately 2µl of cDNA was added in triplicate for each sample into each well on a 96-well PCR plate. Samples were analysed for IFI27, GAPDH and IL-1β. The details of the primers used are shown in table 9.

Primer Name	**Sequence**
mGAPDH-F	5'-GCCAAGGTCATCCATGACAAC-3'
mGAPDH-R	5'-GGGGCCATCCACAGTCTTC-3'
mGAPDH-FAM	5'-[6-FAM]CTCATGACCACAGTCCATGCCATCACT[TamraQ]-3'
IL-1β	Mm01336189_m1 IL1b (Applied Biosciences)
IFI27	Mm01329883_gH If127 (Applied Biosciences)

Table 9: Primers Used In Taqman Gene Expression.

Master mix containing primers and H_2O was added to each sample to make the final reaction volume 15µl, according to the following protocol,

For GAPDH (1 sample)

H_2O	7.375µl
Forward primer	0.375µl
Reverse primer	0.375µl
Probe	0.375µl
TaqMan® Universal PCR Master Mix	4.5µl
(Applied Biosystems, Cat.no.: 4304437)	

For IL-1β and IFI27 (1 sample)

H_2O	7.75µl
Primer Mix	0.75µl
TaqMan® Universal PCR Master Mix	4.5µl
(Applied Biosystems, Cat.no.: 4304437)	

PCR Plates were then sealed and run on a TaqMAN PCR Machine on the following program,

1. 50°C for 2 minutes – Segment 1
2. 95°C for 10 minutes – Segment 2
3. 95°C for 15 seconds – Segment 3
4. 60°C for 1 minute – Segment 3

Segment 3 is repeated for 40 cycles.

Upon finishing, data was analysed on an accompanying computer and used to generate the graphs shown in Results.

Chapter 3: Results

3. Results

3.1 Characterisation Of An *In Vivo* Model For The Skin Disease Psoriasis

3.1.1 Expression Of PPARδ In Human Skin

Previous work by our lab had established and reconfirmed the upregulation of PPARδ mRNA in psoriatic skin lesions by analysis of global expression in two independent cohorts, totalling 58 paired lesional and non-lesional data sets, revealing that PPARδ is upregulated whereas the other two PPAR isoforms, α and γ, are downregulated (Romanowska et al 2010) (see Introduction). To verify the localisation of PPARδ expression and distribution on the protein level in both normal human skin and psoriasis-affected skin, immunohistochemistry was performed on skin sections from both groups. This revealed that in normal skin, PPARδ was predominantly expressed in the cytosol in the lower epidermis (Figure 3.1 A). In contrast, in psoriasis-plaques, PPARδ is additionally strongly expressed in the nucleus and in the upper spinous layer (Figure 3.1 B). This was highly reproducible, being observed in eight different patient samples (supplementary figures of (Romanowska et al 2010)). Figure 3.1C shows the negative control, confirming that the expression of PPARδ observed is specific. These results confirm the previously noted overexpression of PPARδ in psoriasis. Based on the observed maximal nuclear expression in the upper spinous layer, these results suggest that PPARδ may regulate late epidermal differentiation.

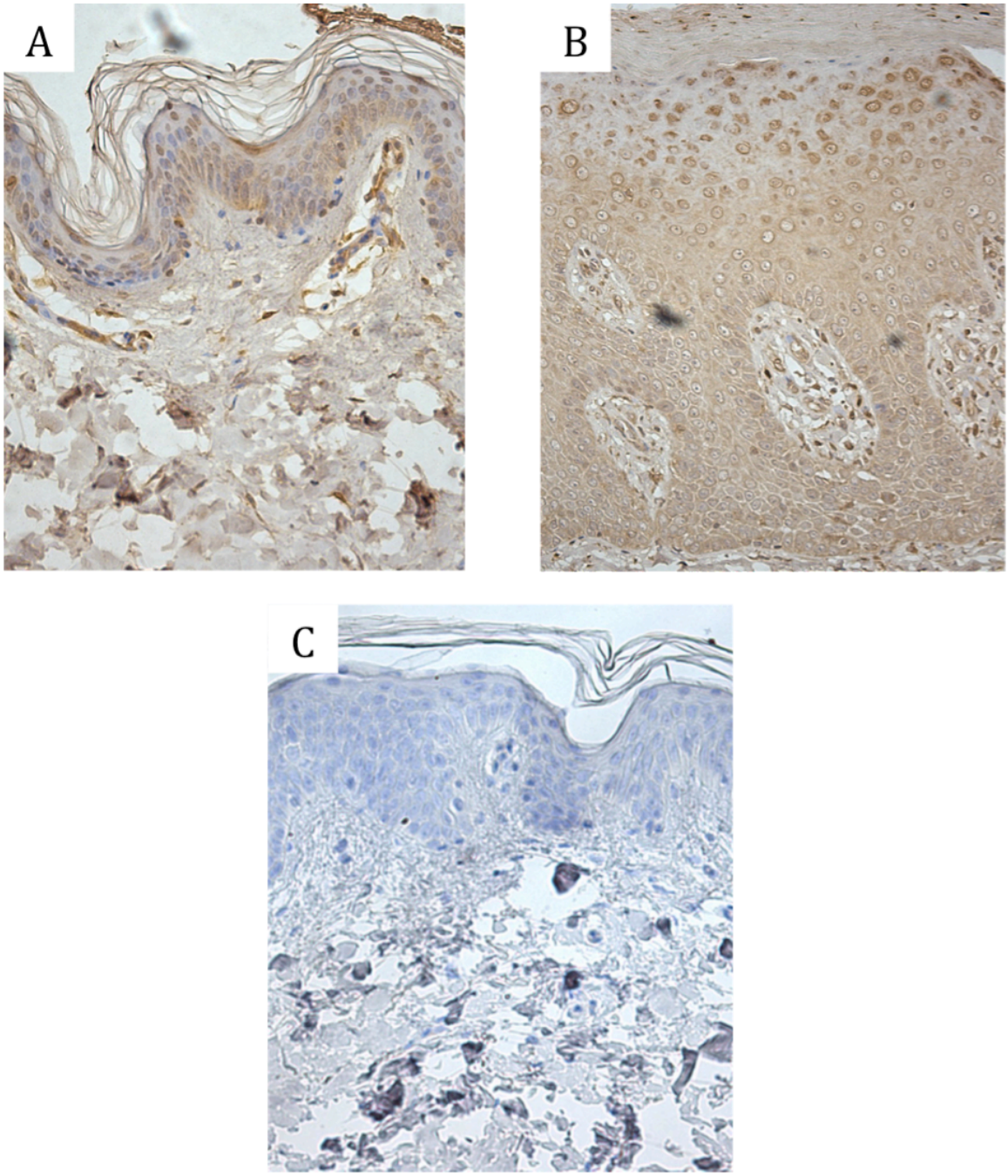

Figure 3.1: Immunohistochemistry Shows Cytosolic PPARδ Expression In Normal Human Skin And Is Over-Expressed In The Nucleus In Psoriatic Skin. *Immunohistochemical PPARδ staining of paraffin-embedded human skin. Normal human skin (A) shows PPARδ expression in the lower epidermis, with a cytosolic expression pattern. Psoriasis (B) shows nuclear accumulation of PPARδ in the spinous layer, with some peri-nuclear staining, the reason for which is unknown. The negative control (C) confirms the specificity of staining. Magnification 200x.*

3.1.2 Expression System Design For *In Vivo* Targeted Overexpression Of PPARδ

In order to characterise the function of PPARδ in upper epidermal layers, our group took advantage of an already available transgenic mouse strain expressing PPARδ. This transgenic line expresses human PPARδ under the control of the rat CYP1A1 enhancer resulting in specific expression of the transgene in the skin, as follows (schematic shown in figure 3.2). Within the CYP1A1 enhancer, two regulatory elements are contained. First, there is a DXE/XRE sequence cluster responsive to binding of the aryl-hydrocarbon receptor (AhR). For AhR to bind DXE/XRE, it must in turn be activated by specific ligands, such as indole-3-carbinole (I3C). Thus, drugs such as I3C can induce transgene expression in tissue such as liver, which was the original purpose of the model. Second, apart from transactivation by AhR, the same enhancer is constitutively active in sebaceous glands (Rowe et al 2008). This AhR-independent selective high-level transgene expression is mediated by a cis-acting G/C rich enhancer element in the CYP1A1 promoter, which has been previously found to drive strong sebaceous gland expression (Kaufman et al 2002). Thus, under untreated conditions, PPARδ transgenic mice express the transgene specifically in the sebaceous glands (figure 3.2). Upon administration of a PPARδ specific ligand, GW501516, this expression becomes more widespread in the overlying epidermis. The mechanism underlying this secondary induction is the known activity of PPARδ in stimulating the differentiation of sebocytes (Michalik and Wahli 2007) and delivery of sebum to the skin (Trivedi et al 2006). The sebum produced, in turn, is rich in ligands capable of activating AhR, such as 15-epi-lipoxin A4 (LXA4) and 5,6-dihydroxy-7,9-trans-11,14-cis-eicosatetraenoic acid (5,6 – DiHETE). Once activated by these ligands, AhR is therefore able to transactivate the expression of the PPARδ transgene in the epidermis via an interaction between the AhR and the DXE/XRE responsive element in the

CYP1A1 enhancer. The net effect is an inducible expression of PPARδ in the epidermis upon administration of the PPARδ-activating ligand GW501516, as summarised in figure 3.2.

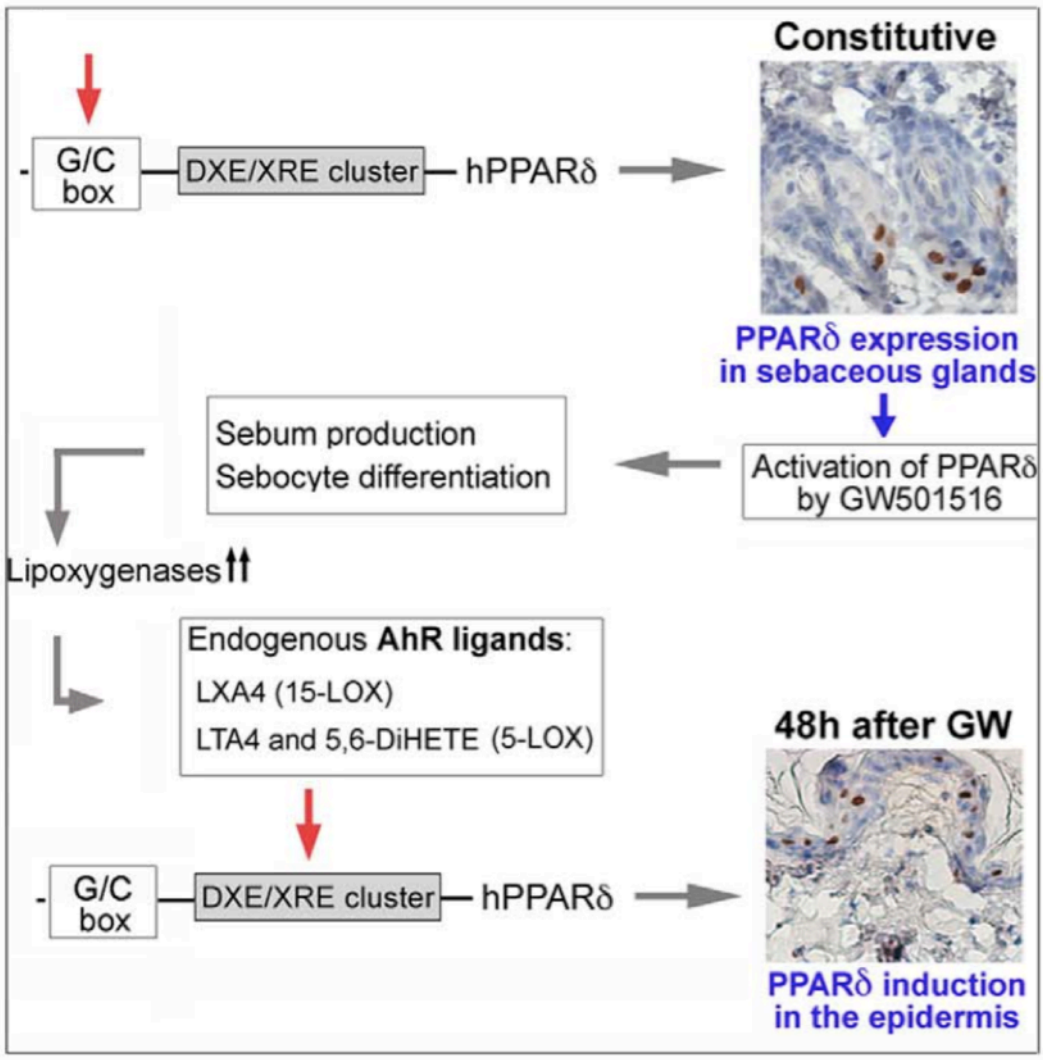

Figure 3.2: System For Inducible Skin-Targeted PPARδ Expression In Vivo And Activation Of Transgene In Murine Epidermis. *Schematic representation of PPARδ transgenic induction system. PPARδ is highly and constitutively expressed in the sebaceous glands of these mice due to the presence of a highly conserved G/C rich box that confers homology to genes specific for the sebaceous glands. Using GW501516 to activate PPARδ, the sebocytes differentiate and produce sebum, which contains endogenous ligands for the AhR which is fused to the CYP1a1 promoter. This drives the expression of PPARδ throughout the epidermis. Modified from (Romanowska et al 2010).*

3.1.3 Detection Of Transgene In Animal Model

Following the generation and expansion of the transgenic line, mice were genotyped to determine transgenic status. Ear tags were taken from each mouse in the litter and the DNA extracted (see Methods and Materials). Once extracted, the DNA was then subjected to PCR analysis using primers specific for the transgene inserted in the mice. The PCR product was analysed by gel electrophoresis. Transgenic status was determined by the presence or absence of a 1.4kb band shown on the gel (figure 3.3), verified by a positive and negative control. Those expressing a positive result were deemed transgenic and those negative were deemed wild type. Based on these results, mice expressing the transgene were selected for experiments, identified by their ear tag status.

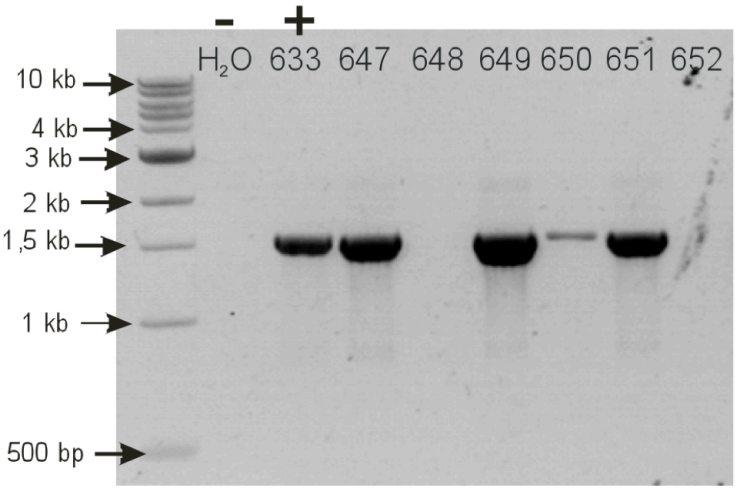

Figure 3.3 PCR Amplification Of PPARδ Transgene From Ear Skin DNA Distinguishes Murine Trangenic From Non-Transgenic Littermates. *20ng of DNA extracted from mouse ear tags was subjected to PCR analysis with primers recognising human PPARδ. The gel shown is typical for all mice analysed. All mice positive for transgene show a band approx. 1.4kb. Note sample 650, which is not positive and is most likely due to contamination, as it is a fainter band compared to the other positive results shown.*

3.1.4 Specificity Of PPARδ Expression

Once the expression of PPARδ following induction in transgenic mice had been verified, the "leakiness" of the induction system was then investigated, i.e. if there was PPARδ expression elsewhere in the mice. A variety of organs were checked, specifically ones that are known to express PPARδ in humans. The colon, liver, muscle, spleen and lymph node of control transgenics and induced transgenics were subjected to immunohistochemistry analysis, staining for human PPARδ. As shown in figure 3.4, there was no staining for PPARδ in any of the organs studied, in either control transgenics or induced transgenics. This confirmed that the induction system described above was tightly controlled, with constitutive expression only occurring in the sebaceous glands and following initiation with GW501516, in the epidermis.

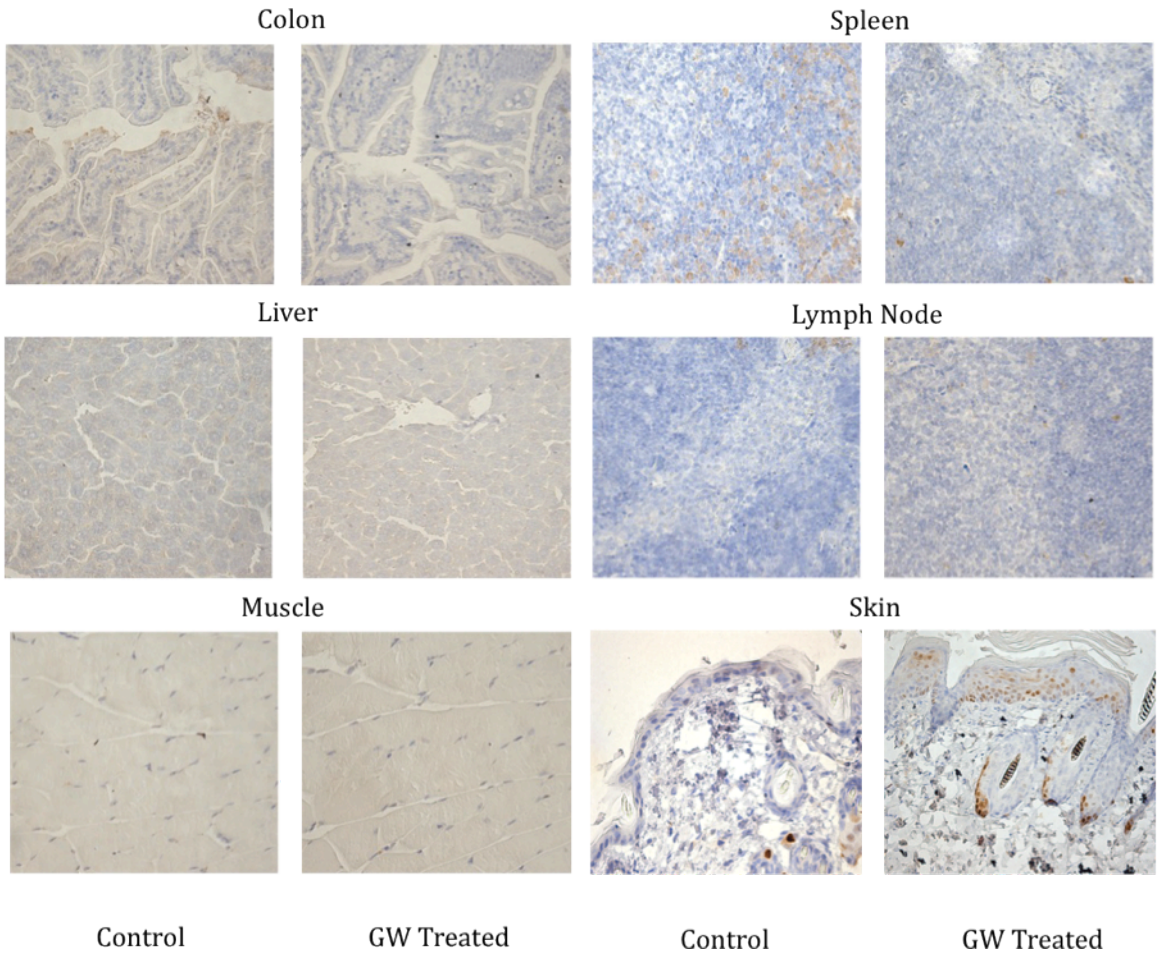

Figure 3.4 Immunohistochemistry Staining Of Colon, Liver, Muscle, Spleen And Lymph Node Reveal Specific PPARδ Expression Is Only Observed In Skin Following Activation. *Immunohistochemistry of paraffin-embedded organ sections. Sections were stained for PPARδ to verify the specificity of the expression system used in the model. As can be observed, there is little to no positive staining for PPARδ in all organs examined. Skin is included as a positive control. Magnification 200x.*

3.1.5 Phenotype Of PPARδ Transgenic Mice Upon Induction

Mice were induced with GW501516, a specific PPARδ agonist, diluted in powdered diet at a concentration of 0.003% for 2 weeks. The results revealed a specific skin phenotype in mice treated with GW501516 diet compared to control mice. GW501516 treated mice showed scaling, inflammation and skin thickening (figure 3.3). Hyperkeratosis (indicated by skin roughening) and hair loss was observed, primarily in regions that are subjected to mechanical friction such as the abdomen, paws and chin (figure 3.3). The development of these changes on sites of mechanical friction is reminiscent of plaque formation in patients with psoriasis. Some mice also developed psoriasis-like plaques on the back, however skin changes in the majority of mice were limited to scaling on the dorsal skin. A minor phenotype that is also observed in mice induced with GW501516 is weight loss and is observed in all mice studied. Since this is a well-documented metabolic effect of GW501516, this phenotype was not further investigated (Harrington et al 2007, Tanaka et al 2003).

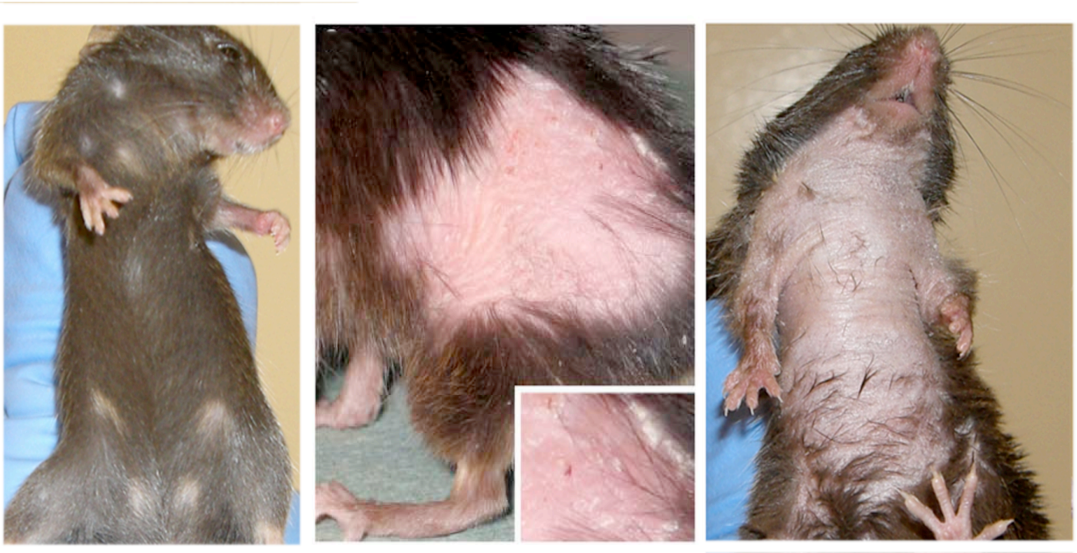

Figure 3.5: Following Induction, PPARδ Transgenic Mice Show A Psoriasis-Like Phenotype. *Clinical photographs detailing the phenotype of PPARδ transgenic mice after induction with GW501516 for 20 days. These images are representative of all transgenic animals treated with GW501516. Note in the middle panel the formation of a "psoriatic" plaque on the back skin. Mice were manually restrained which creates artificial tightening of the skin.*

Histologically, mice induced with GW501516 show marked epidermal thickening compared to non-treated controls (figure 3.6). This is accompanied with dilation of dermal blood vessels and the increased abundance of lymphocytes. In contrast to psoriasis in humans, the granular layer is still present in the skin of these mice. This can be explained by the known role of PPARδ in epidermal differentiation (Schmuth et al 2008). Although the basic phenotype shows 100% penetrance, there is variability in the degree of epidermal hyperproliferation observed in mice induced with GW. The most likely explanation for this is biological variability. Even though the used transgenic strain is "inbred" this only means near-identity in the HLA-system. The remaining genome continues to differ between individual mice. In addition, body weight may be a factor, i.e. if the starting body weight is higher then mice will require more GW501516

before they exhibit a phenotype and vice versa. As male C57 Black 6 mice are larger than female mice, gender might be a factor however no systematic difference between male and female mice has been found in this model. Whatever the cause, it appears that mice have natural variation in the degree of phenotype observed.

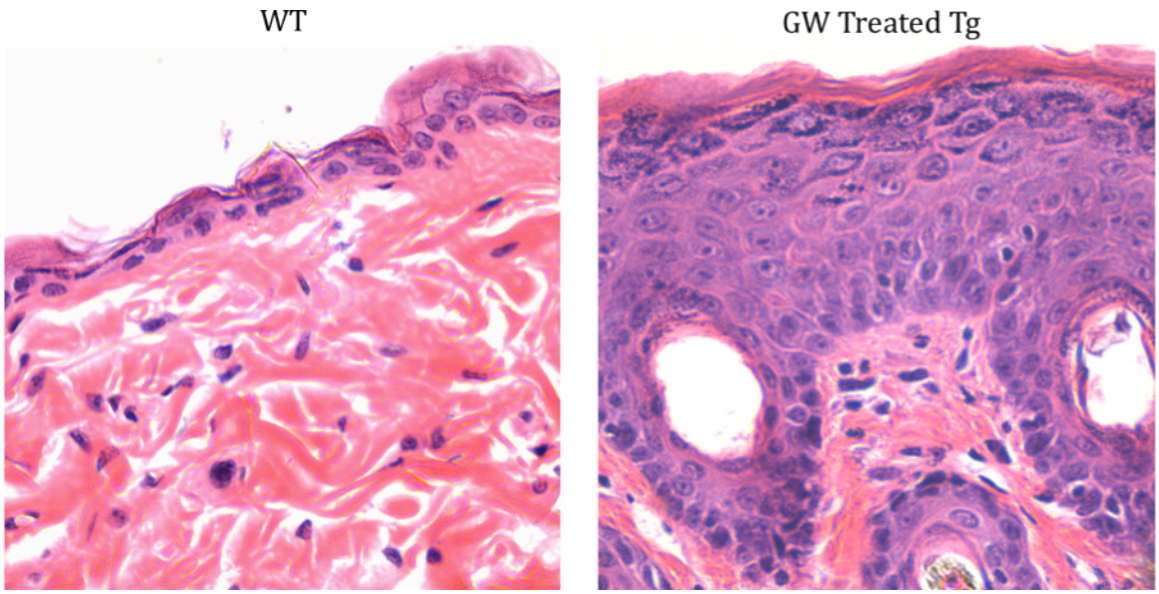

Figure 3.6: H&E Histology Show Epidermal Hyperplasia In GW-Induced Mice Compared To Wild-Type Controls. *Comparison of H&E histology between WT and GW-treated transgenic (tg) skin. The right panel shows skin histology following induction for 20 days with GW501516.*

3.1.6 Assessment Of PPARδ Expression In Murine Skin

As described above, upon treatment with the specific PPARδ agonist GW501516, mice developed a psoriatic-like phenotype (figure 3.3). To assess the specificity of the induction system on PPARδ overexpression, immunohistochemistry was performed on PPARδ transgenic mice at rest, induced and compared with wild type littermates. As expected, there was no transgenic PPARδ expression in wild type skin (figure 3.7B),

however in PPARδ transgenics (tg) not induced with GW501516, there was staining of PPARδ observed in the sebaceous glands (figure 3.7C), as described, above (section 3.1.2). Upon induction with GW501516 for 7 days the expression of PPARδ became more widespread in the epidermis. The epidermis was also thickened due to the stimulatory role of PPARδ on keratinocyte proliferation (figure 3.7D).

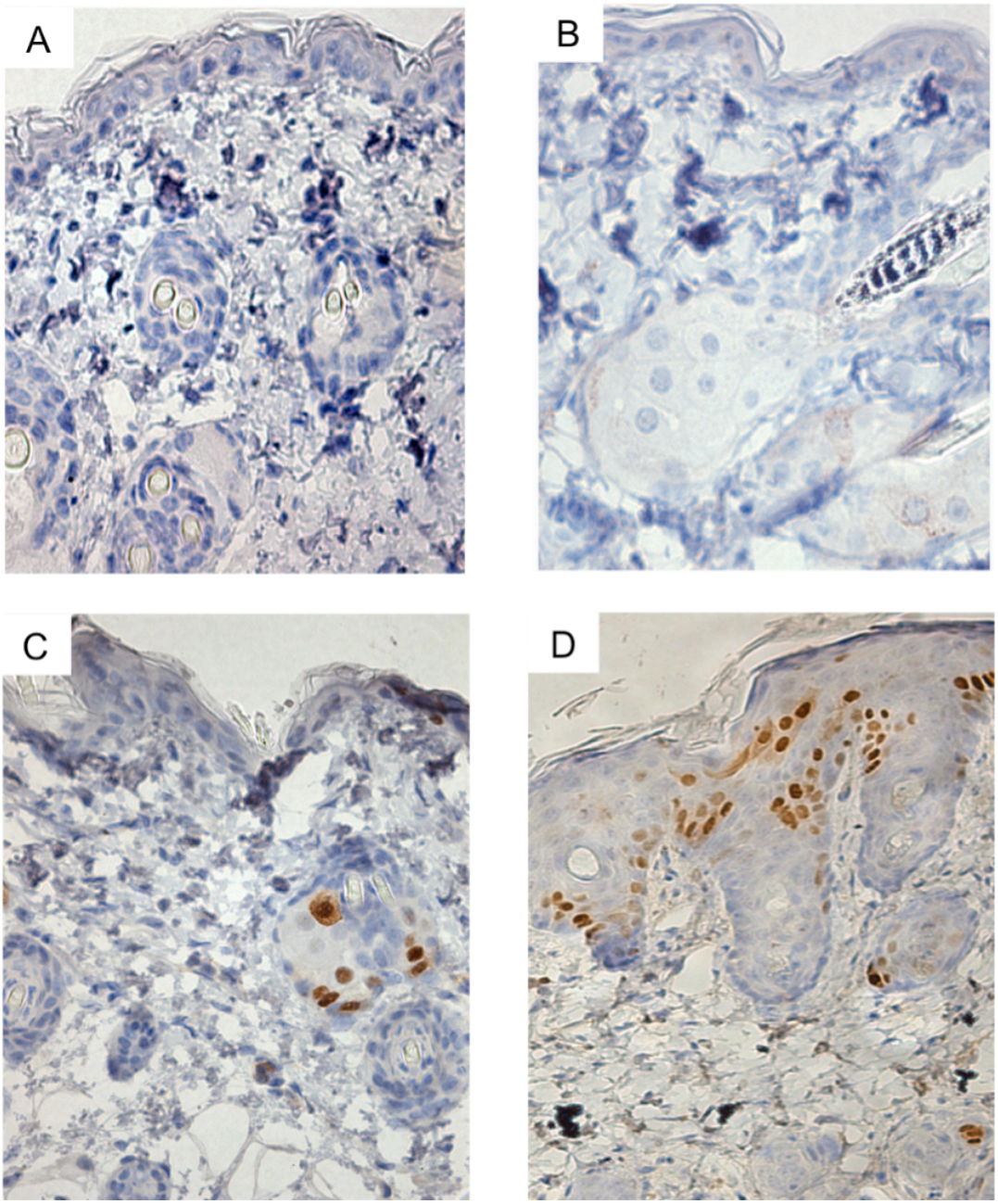

Figure 3.7: Immunohistochemical Assessment Of PPARδ In Transgenic Murine Skin Revealed Expression In Sebaceous Glands Under Resting Conditions And Widespread Expression In GW-Induced Mice. *Immunohistochemistry for PPARδ in murine skin. A shows negative control (PPARδ, non-induced, transgenic abdominal skin, treated with secondary antibody only), B panel shows staining for PPARδ in C57 black 6 wild type (WT) skin, C shows PPARδ staining in PPARδ transgenic and D shows PPARδ staining in PPARδ transgenic induced with GW501516 for 7 days. Magnification x200.*

To assess how quickly PPARδ expression was switched on in response to induction with GW501516, mice were induced for a total of 7 days with 0.003% GW501516 diluted in powdered diet and various time points taken. The time points chosen were 0hrs, 48hrs, 96hrs and 7 days. As early as 48 hours following induction with GW501516, protein translation was observed in the epidermis (figure 3.8, middle panel). At 96 hours, epidermal changes were already under way with the marked thickening of the epidermis and the expression of PPARδ becoming yet more widespread. The expression of PPARδ in the sebaceous glands was constitutively high, as outlined above, with strong positive staining observed (figure 3.8, upper left panel). Notably, after 7 days of induction, the epidermis was markedly thickened compared to skin at 0 hours after induction, with PPARδ expression observed throughout the epidermis. With this change in the epidermis, a phenotype was also observed in the mice similar to that shown in figure 3.5.

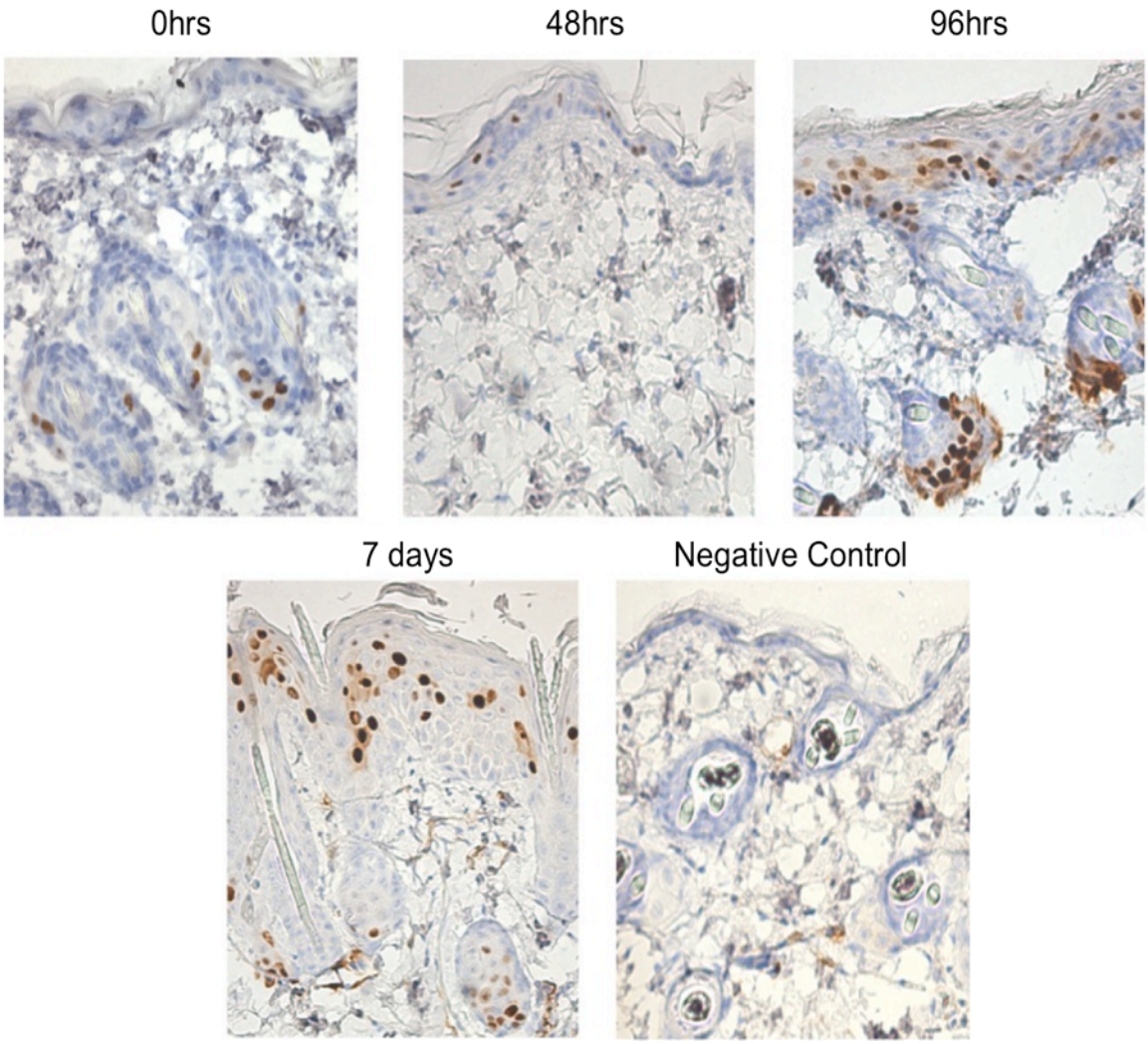

Figure 3.8: Timecourse Induction Reveals Expression Of PPARδ In Skin As Early As 48hrs Following Induction By GW501516. *Immunohistochemical analysis of PPARδ expression at differing time points following GW501516 induction. From left to right, top row, 0hrs, 48hrs, 96hrs, bottom row, 7 days after induction and the negative staining control (PPARδ non-induced transgenic abdominal skin, with secondary antibody only). Magnification 200x.*

3.1.7 Characterisation Of Immunological Changes In PPARδ Mice

To further compare the phenotype of the mice with respect to known alterations in psoriasis, the changes in the levels of immune cells recruited to psoriatic skin were measured by immunohistochemistry. As outlined in the Introduction various immune cells are recruited to the skin in psoriasis upon the induction of psoriatic plaque formation resulting in inflammation. The main cells recruited are $CD4^+$ and $CD8^+$ T cells as well $CD11c^+$ dendritic cells. The staining of CD31, which is a marker for endothelial activation, suggesting the stimulation of angiogenesis, a key feature of psoriasis, was also investigated. All these immunohistochemistry stainings were performed on frozen O.C.T embedded skin sections from both control transgenics and induced transgenics. In control sections, there are some $CD4^+$ cells detectable in the dermis; with the level of these cells increasing in the dermis of transgenic induced with GW501516 (figure 3.9, top row). In contrast, $CD8^+$ cells were barely detectable in control skin, however there was a modest increase in their levels following induction, particularly in the epidermis (figure 3.9, second row). $CD11c^+$ dermal dendritic cells were present in low levels in control mice, with numbers increasing in GW501516-treated mice (figure 3.9, third row). However, there was an absence of $CD11c^+$ Langerhans cells, which correlates with the impaired migration observed in psoriasis (Cumberbatch et al 2006). Finally, expression of CD31, a marker for endothelial cell activation, was increased in GW501516-treated mice compared to the levels observed in control mice (figure 3.9, bottom row). All of these changes were analogous to the type of infiltrate seen in psoriasis plaques.

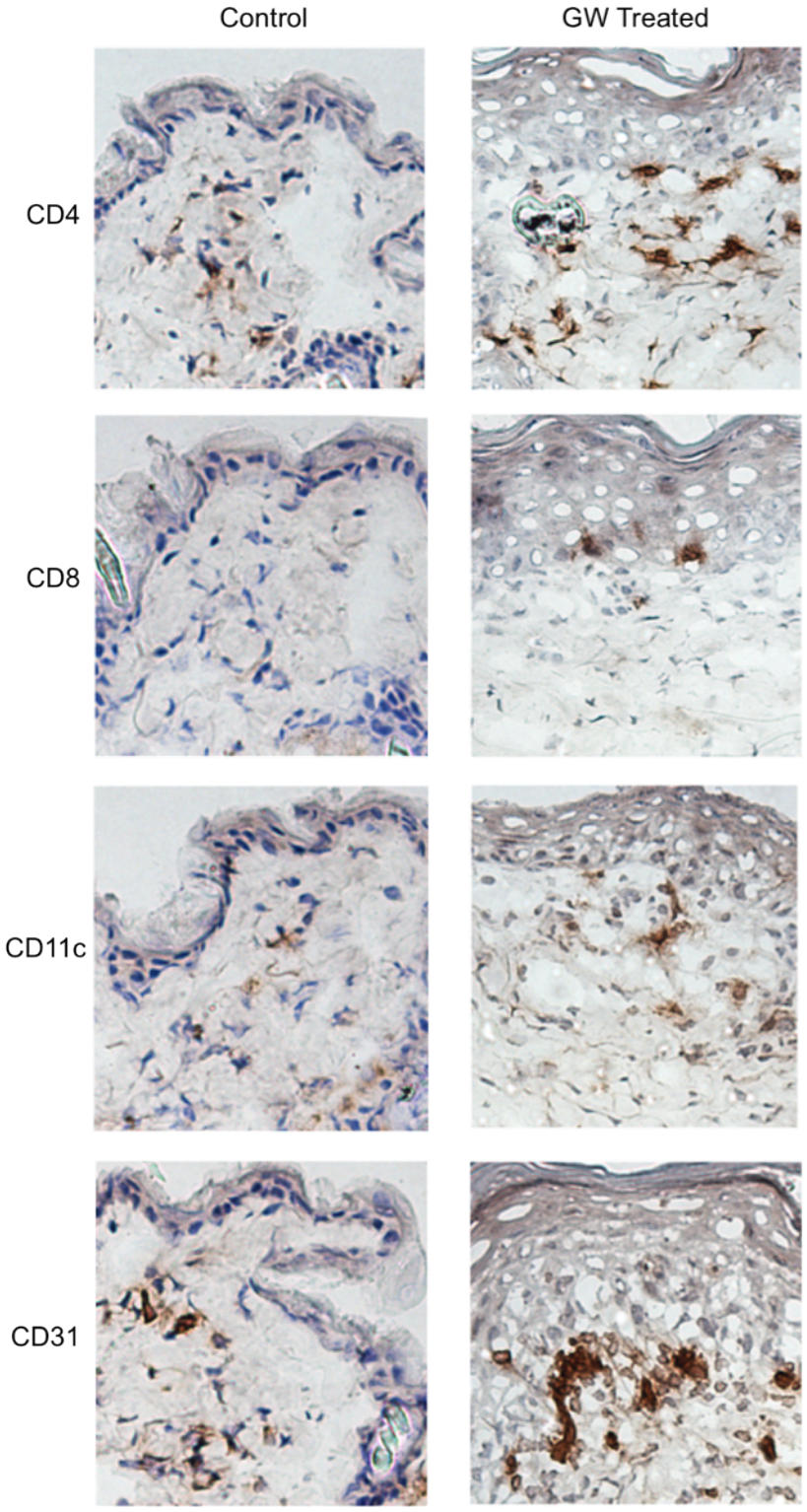

Figure 3.9: GW501516 Stimulates An Increase In CD4$^+$, CD8$^+$, CD11c$^+$ And CD31$^+$ Cells In Skin of GW-Induced Transgenic Mice. *Changes observed in CD4, CD8, CD11c and CD31 expressing cell numbers in control and GW501516- treated mice. Images are representative and taken at 200x magnification.*

3.2 Characterisation Of STAT3 Involvement In PPARδ *In Vivo* Model

3.2.1 Expression Of STAT3 And Phospho-STAT3 *In Vivo*

As detailed in the Introduction, STAT3 is highly activated in psoriasis, indicated by the presence of phosphorylated STAT3 in the epidermis (Sano et al 2005). Accordingly, we investigated the role of STAT3 in our psoriasis-like animal model. Transgenic mice were induced with GW501516 for 2 weeks, and then sacrificed and skin biopsies harvested. In parallel, non-induced mice were used as controls. Whole cell protein extracts from both non-induced and induced mice were extracted using so-called Klose buffer as detailed in Methods. These samples were then subjected to Western Blot analysis, which revealed that STAT3 expression was unchanged in both non-induced transgenic mice and induced mice (Figure 3.10). However, levels of STAT3 phosphorylated at Tyr705 ("phospho-STAT3"), the activated form of STAT3, were induced with GW501516. When densitometry was applied to western blot results, the difference in the protein level of phospho-STAT3 and STAT3 was confirmed, showing no change in the level of either protein in the control animals but in the mice induced with GW501516, the levels of phospho-STAT3 being greatly upregulated.

Interestingly, there were two bands detectable when probing a blot of whole cell protein skin extracts with an anti-STAT3 antibody. These bands were around 4-5 kDa apart, with the upper band being 86 kDa in size and the lower around 79 kDa. This double band corresponds to the two isoforms STAT3-α (upper) and STAT3-β (lower), respectively, as previously described (Maritano et al 2004). Specific hyperphosphorylation of STAT3 α, as seen in the figure, was observed in three other western blots of whole cell protein skin extracts probed with this antibody.

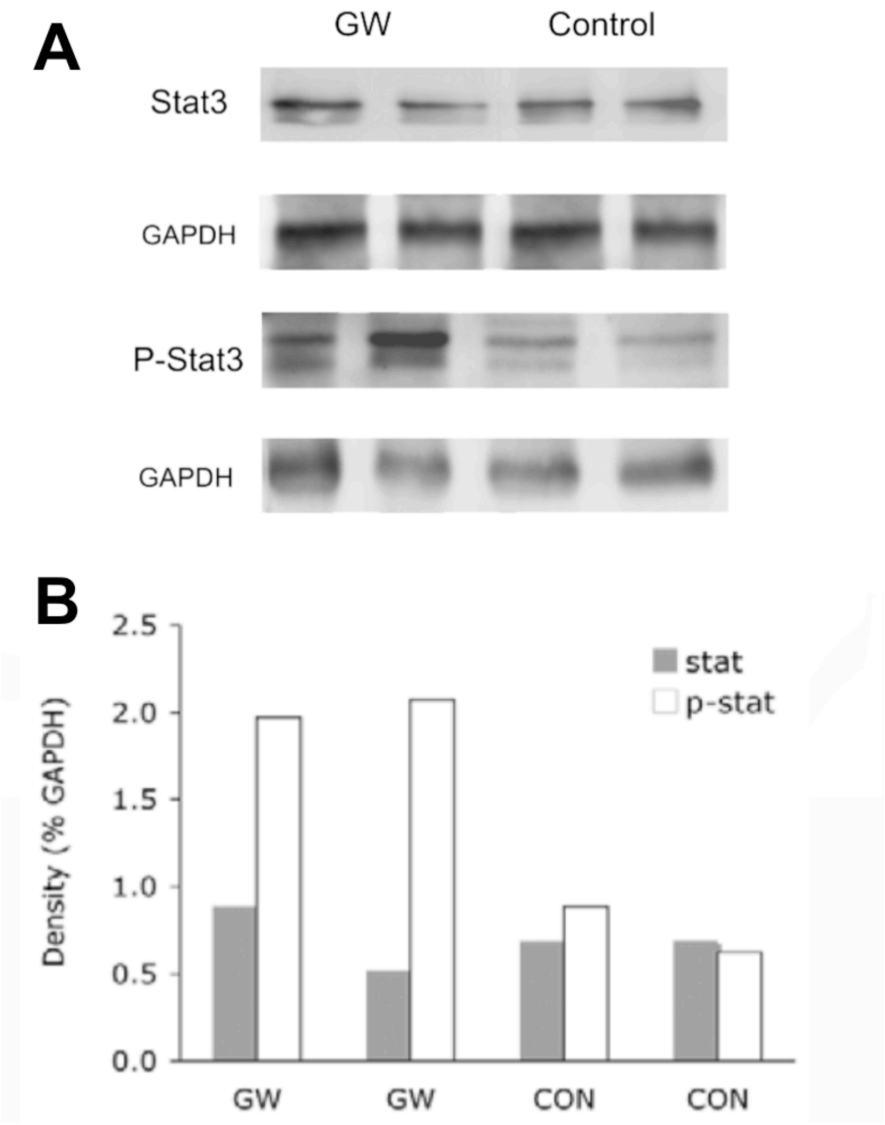

Figure 3.10: Western Blot Analyses Show Hyperphosphorylation Of STAT3 In GW-Induced Mice. **A** *shows western blot analysis of whole skin extracts probed with STAT3 and phospho-STAT3 (P-STAT3).* **B** *shows densitometry analysis of western blot results.*

3.2.2 Localisation Of Phospho-STAT3 In The Skin Of PPARδ Transgenic Mice Following Induction

In order to determine the localisation of phospho-STAT3 in lesional skin from induced mice, an immunoflurorescence protocol was established in the laboratory. Analysis of the sections revealed that there was an increase in the presence of phospho-STAT3 in GW501516-induced mice only (figure 3.11). The expression of phospho-STAT3 in non-induced mice was barely detectable, predominantly due to the skin only being a few cell layers thick, therefore making the localisation of phospho-STAT3 difficult to detect in frozen sections. The expression of phospho-STAT3 was predominantly localised to the nuclei of the suprabasal layer in the lesional skin of induced mice. To verify the specificity of staining observed in all sections, phospho-STAT3 was competed with a blocking peptide. DAPI staining was also performed to verify the nuclear localisation of phospho-STAT3.

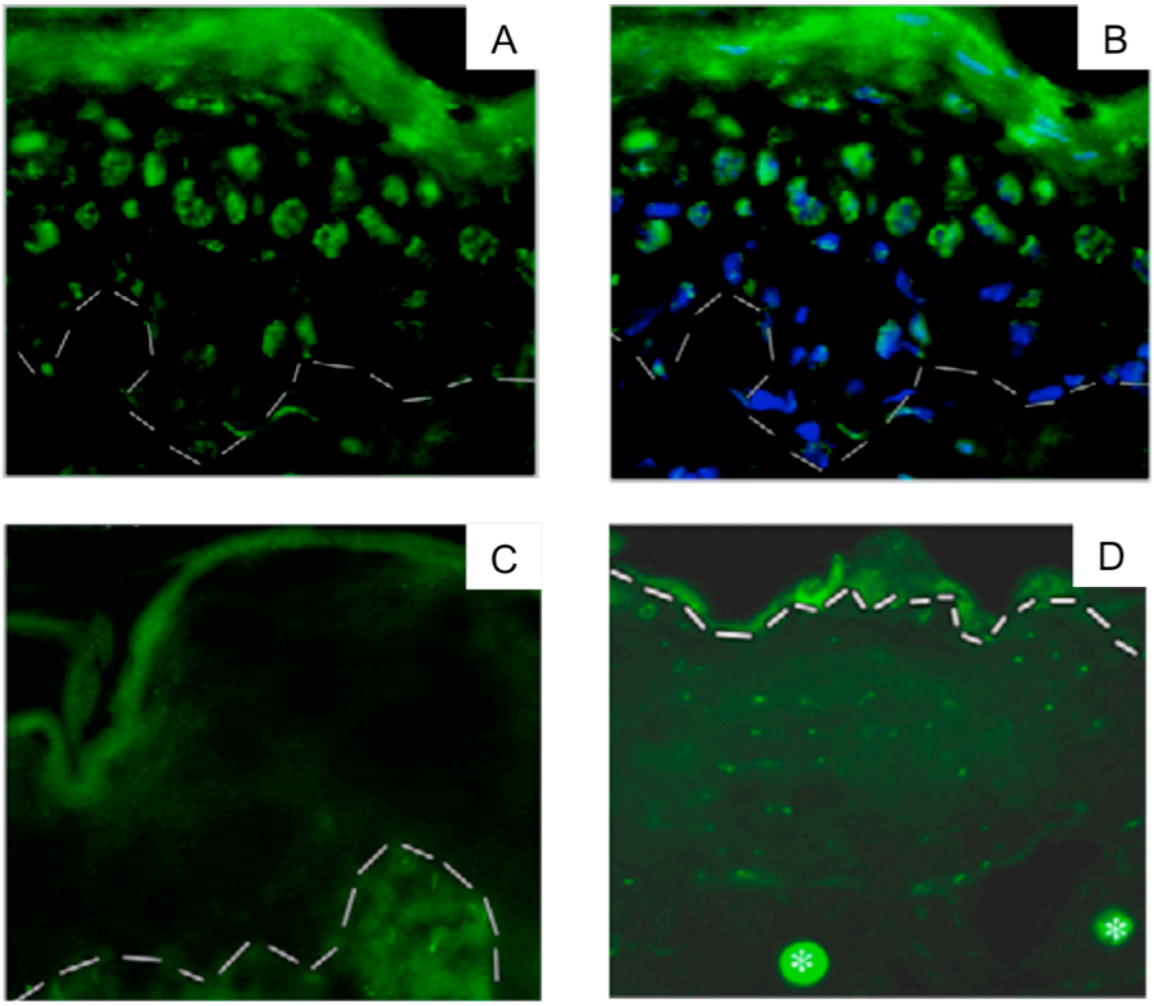

Figure 3.11: Immunofluorescence Shows That Phospho-STAT3 Is Localised To The Nucleus In The Suprabasal Epidermis In GW-Induced Mice. *Immunofluorescence of 5µm frozen sections from induced and non-induced mice treated with anti-phospho-STAT3 or competed with blocking peptide. Upper panels show GW induced mice, with (A) and without (B) DAPI staining, shown to verify nuclear localisation. Lower panels show competition with blocking peptide (C) and non-induced skin (D). The white dashed line represents the dermal-epidermal barrier. *=hair shaft. All images taken at 400x magnification.*

3.2.3 Role Of STAT3 In Disease Progression *In Vivo*

In order to determine whether STAT3 activation contributes to progression of psoriasis-like disease in PPARδ mice, transgenic mice were induced with GW501516 in the absence or presence of inhibition of STAT3 phosphorylation. As shown in figure 3.12, concurrent treatment with the JAK2 inhibitor substantially inhibited the development of epidermal hyperproliferation and inflammatory changes. This suggests that STAT3 phosphorylation may contribute to the development of skin disease induced by PPARδ activation. However, due to the lack of full inhibition of development of the phenotype, the phenotype observed in these mice cannot solely be attributed to activated STAT3. Rather, other molecular players appear to be at work that continue to drive the psoriatic phenotype.

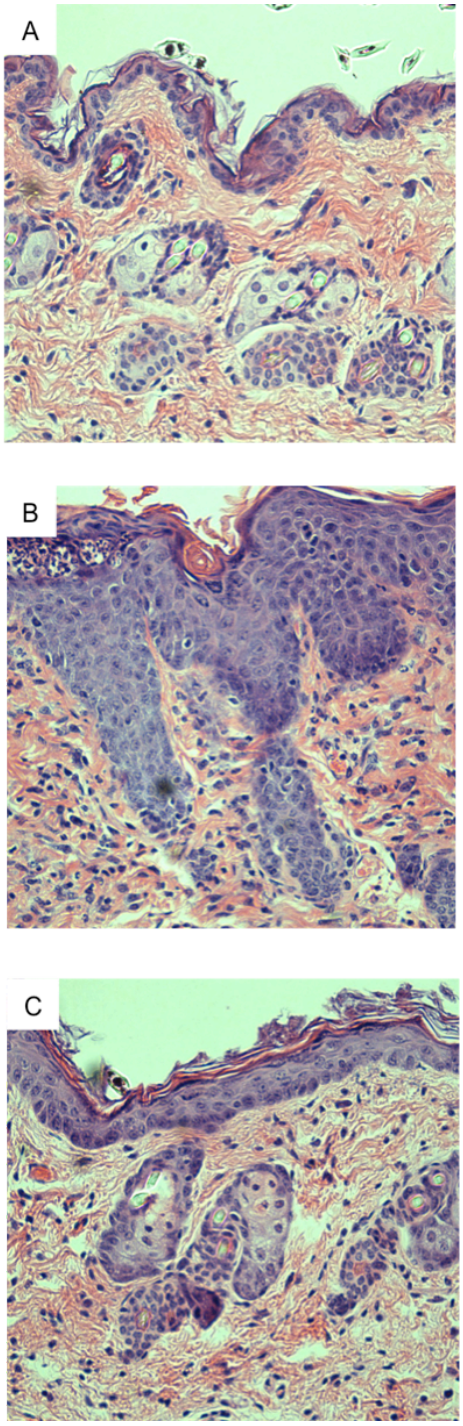

Figure 3.12: Treatment Of GW-Induced Mice With JAK2 Inhibitor, WP1066 Partially Inhibits Development Of Epidermal Hyperplasia. *H&E histology of untreated skin (A), GW501516-treated skin (B) or GW501516 + JAK2 inhibitor (C). Treatment with WP1066 partially inhibited the development of disease phenotype. Images are representative, taken at 200x magnification.*

3.2.4 Effect Of JAK2 Inhibition On Gene Expression

A major characteristic of psoriasis is that a group of genes termed the "interferon response genes" are upregulated. However, in this particular transgenic model, the interferon response genes are actually downregulated (Romanowska et al 2010). Notably, it has previously been shown that STAT3 represses the exact same set of genes *in vivo* (Dauer et al 2005). Therefore, we hypothesised that STAT3 activation underlies the repression of interferon response genes, such as IFI27, in this model as well. If so, their repression should be reversible by JAK2 inhibition. Indeed, JAK2 inhibition did show a significant effect on the reversal of IFI27 downregulation (Figure 3.10), IFI27 being chosen as one of the most strongly downregulated and well characterised IFN target genes, thus it partially rescued the expression of IFI27 back to levels comparable to those observed in control mice. In contrast, JAK2 inhibition had no effect on IL-1β, which is situated in another inflammatory pathway, thereby suggesting that STAT3 activation by JAK2 regulates interferon response genes, but not other inflammatory circuits. This result indicates that the interferon-signalling inhibition observed in these mice is possibly regulated by STAT3, which has been termed the "anti-inflammatory response" (Murray 2006).

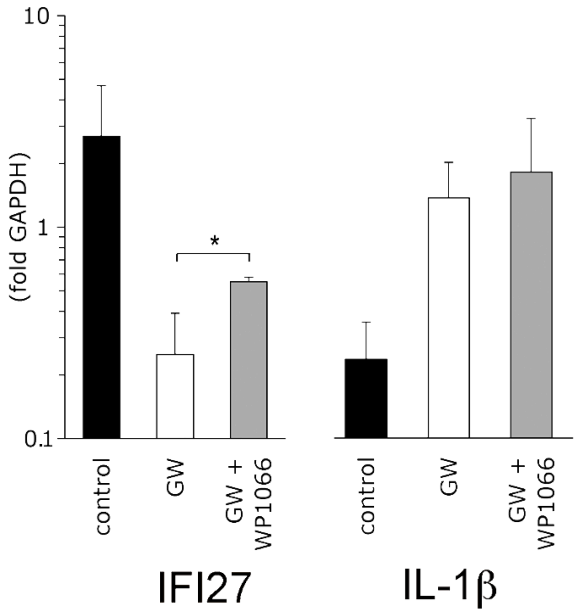

Figure 3.13: JAK2 Inhibition Results In Partial Rescue Of IFI27 Gene Expression With No Effect On Other Inflammatory Pathways. *TaqMAN gene expression analysis of IFI27 and IL-1β from whole skin of untreated mice (black column), GW-treated mice (white column) and GW-treated mice + JAK2 inhibitor (grey column) respectively. (n= 3 mice per group)* ******* *= $p<0.05$.*

Chapter 4: Discussion

4. Discussion

4.1 Generation Of *In Vivo* Model Of Psoriasis

The transgenic mouse model characterised in the course of the present thesis exhibits a number of unconventional features meriting discussion. In order to test whether activation of PPARδ by itself is sufficient to trigger psoriasis-like skin changes *in vivo*, one could have chosen a different strategy. In the present case, however, a model was already available, in which human PPARδ had been placed under the control of the CYP1A1 enhancer in order to allow inducible expression in the liver upon administration of AhR activating chemicals. Strikingly, and completely unexpected, this model also afforded a skin-targeted expression of PPARδ. In addition to AhR-mediated inducible expression in the skin, a previously unnoticed enhancer element conferred very high constitutive expression in the sebaceous glands, thereby creating a model wherein PPARδ was continuously expressed in the sebaceous glands. Two additional fortuitous elements were required to turn this sebaceous-specific expression pattern into an epidermis-specific inducible model. First, the fact that, in our case, the transgene, (PPARδ), happens to be a natural activator of sebaceous gland differentiation and sebum production. Thus, simply adding an activating ligand triggered sebum production. Second, sebaceous differentiation, sebum production, and the concomitant upregulation of lipoxygenases resulted in the delivery of bioactive lipids to the epidermis, which in turn transactivated the AhR in the epidermis. Obviously, this system was highly unorthodox and it could be argued that it is less “clean” than using a standard epidermis – specific controlling promoter. However, a number of key features single out this particular model as highly appropriate to model PPARδ activation in psoriasis. First, the system is very tightly controlled: no inducible expression is seen in

any other tissue outside the skin. Second, the system allowed use of a single chemical, GW501516, to achieve both transgene induction and ligand-activation of PPARδ, which massively reduced cost and animal numbers by obviating the need for more control groups. Third, the expression pattern in the epidermis was distinctively patchy (see figure 3.7), which is very similar to the distribution of PPARδ in the nuclei of the spinous cell layer in psoriasis plaques. A conventional suprabasal-active promoter (e.g. loricrin, involucrin) by contrast would produce a more linear expression pattern and, in addition, not allow inducibility. Thus, although a bona fide discovery, the present transgenic model is highly suitable to study the effect of PPARδ in the epidermis.

4.2 PPARδ And Inflammation In The Skin

Psoriasis is predominantly an inflammatory skin disease. To date, PPARδ has been implicated with a role in inflammatory processes. However, there is a controversial debate as to whether it is pro-inflammatory or anti-inflammatory, with evidence reported for both (Barish et al 2008, Hall and McDonnell 2007). Based on the results presented here, it appears that PPARδ plays a pro-inflammatory role in psoriasis (Pollock et al 2010, Romanowska et al 2010). PPARδ induces a specific IL-1 signalling module both in psoriasis and PPARδ transgenic mice, which involves pro-inflammatory mediators such as IL-1β, which has been shown to stimulate Th17 differentiation, a major characteristic of psoriasis (Kryczek et al 2007). This IL-1 signalling module also contains anti-inflammatory mediators, such as IL-1F5 in fibroblasts, which has been shown to inhibit skin disease (Blumberg et al 2007, Magne et al 2006). In addition, the IL1 receptor antagonist (IL1RA) has also been shown to be part of this IL1 module, is a direct target of PPARδ (Chong et al 2009), and is upregulated in psoriasis (Debets et al

1997). Therefore, the role of PPARδ cannot solely be described as pro- or anti-inflammatory.

4.3 Similarity To Human Disease

The data presented here confirm that activation of PPARδ is enough to trigger inflammatory changes in the skin similar to psoriasis. PPARδ mice showed several histological and immunological changes that are synonymous with the human disease. As early as 7 days following induction, mice showed enhanced epidermal thickening, dilation of dermal blood vessels and increased lymphocytes in the epidermis. In addition, the influx of $CD4^+$, $CD8^+$ T cells and $CD11c^+$ dermal dendritic cells, expansion of Th17 cells was also abundant in the affected skin of PPARδ mice (Romanowska et al 2010). This verifies that PPARδ mice following induction replicate several of the key phenotypical changes observed in psoriasis patients. In that respect, the immunological changes and STAT3 activation are in line with previous data from our lab on this model which revealed a high level of similarity of gene dysregulation when compared with expression profiling data sets of psoriasis patients. Shown in figure 4.1 is a summary of the top 50 upregulated genes in the PPARδ transgenic mice compared with two independent psoriasis cohorts. These data show that the majority of genes are upregulated in psoriasis and PPARδ transgenic mice (Romanowska et al 2010).

	PPAR β/δ	Psoriasis I	Psoriasis II		PPAR β/δ	Psoriasis I	Psoriasis II
KRT6B	1084	5.1	6.9	GJB6	20	3.8	8.2
LCE3D	7.5	19.1	55.6	WFDC12	19	3.6	12.5
LPO	182	1.1	1	PLA2G4D	18	1.7	2.8
SPRR1B	181	5.6	11.9	CCL20	17	10.1	20.9
S100A8	160	12	21.6	TGM3	17	4.9	8.7
SLC7A11	135	4.9	5.5	GJB2	15	8.5	17.3
IL8RB	120	4.2	4.7	IDI2	14	1	0.9
SERPINB3	112	46.2	160.8	IL1A	13	1.1	1.4
S100A9	99	51.4	127.5	OLAH	13	0.9	0.9
IL1F6	77	4.6	4.7	SLC5A1	12	4.2	13.8
AKR1D1	72	1	1	PSORS1C2	11	1.6	2.7
CHI3L1	52	1	1	CASP14	11	1.5	4
KLK6	34	16.5	70.5	RPTN	11	2.3	1.5
SERPINB12	34	1.6	7.8	SLC5A9	10	1	1
LCN2	31	20.3	109.4	SLC28A3	10	1.9	1.5
SPRR2B	29	23.9	91.6	CXCL1	10	8.2	14.4
IL1F8	27	1.5	2.3	SERPINB6	10	1.1	1.3
PCDH21	27	0.3	0.3	GALNT6	9.6	4.4	5.7
DEGS2	26	1.2	2.4	SLC34A2	9.4	1	1
TDH	26	1	1	MAGEA1	9.3	1	1
SLPI	25	2.2	2.4	CD163L1	9.2	1.4	1.2
KRT16	24	25.7	36	FLG	9.1	0.6	0.9
NMU	23	1.3	1.8	KCTD4	8.9	2	2.9
TREX2	21	5.1	14.4	IL1F9	8.8	30.8	47.4

Figure 4.1: Congruent Gene Dysregulation In PPARδ And Psoriasis. *Diagram above shows fold change between lesional skin of PPARδ mice and control mice and between lesional and non-lesional skin of psoriasis patients obtained from the GAIN (I) and GSE14905 (II) psoriasis cohorts, respectively. Red shows a fold change of >1.5 and green indicates a fold change of <0.8. The top 50 genes upregulated in psoriasis are shown. Modified from (Romanowska et al 2010).*

4.4 Differences To Psoriasis

Prior to the establishment of this model, it was an open question whether activation of PPARδ in psoriasis is part of the disease process or, on the contrary, represents an attempt of the epidermis to combat the psoriatic reaction. The latter would have been indicated by data suggesting that PPARδ in fact is anti-inflammatory and blocks cell proliferation (Peters et al 2000, Peters and Gonzalez 2009, Romanowska et al 2010). Although the model confirms that activation of PPARδ is sufficient to trigger

inflammatory changes with great similarity to psoriasis, there are two major differences between the transgenic mice and psoriasis. Two clusters of genes were found to be differentially regulated when compared to psoriasis (Romanowska et al 2010). The first of these clusters contains genes that are upregulated in our model but downregulated in psoriasis. This cluster was enriched with markers of late differentiation, such as filaggrin (FLG) and protocadherin 21 (PCDH21). The upregulation of these genes mirrors the continued presence of the granular layer in PPARδ mice, a histological feature that is absent in psoriasis. The known effects of PPARδ on epidermal differentiation can explain this feature. Schmuth et al showed that topical application of a PPARδ agonist increased markers of differentiation *in vivo* (Schmuth et al 2004). Thus, the continued presence of the granular layer in PPARδ mice can be attributed to the prodifferentiation effects that PPARδ exerts on keratinocytes and the epidermis.

The second cluster of genes found to be upregulated in psoriasis but downregulated in PPARδ mice contained genes involved in interferon signalling. This may in fact be related to the increased levels of activated STAT3 in PPARδ mice after induction of disease (section 3.2, also below, section 4.13). To this end, Dauer and colleagues showed that STAT3 represses this group of genes *in vitro* (Dauer et al 2005), thereby possibly explaining why this particular set of genes is downregulated in our model, which expresses an increased level of activated STAT3 in response to phenotype induction with GW501516.

Taken together, activation of PPARδ in the epidermis leads to psoriasis-like inflammatory and immunological changes, which may be in part mediated via STAT3 signalling (see below, section 4.13). On the other hand, the block in terminal differentiation, which is characteristic of psoriasis, does not occur in these mice. Indeed,

the physiological role of PPARδ is to enhance epidermal differentiation (Schmuth et al 2004). It is therefore possible that, in psoriasis, suppression of terminal differentiation and the block in skin barrier repair may in fact lead to the non-terminated upregulation of PPARδ in the upper spinous layer of the epidermis (Romanowska et al 2010), which in turn would explain the chronic nature of the disease clinically. Therefore, genetic variants that block terminal differentiation would be expected to enhance psoriasis risk. This is exactly what appears to be the case with the LCE3 gene variants (de Cid et al 2009). The end result is a feedback loop resulting in the chronic and persistent course of psoriasis, as summarised in figure 4.2.

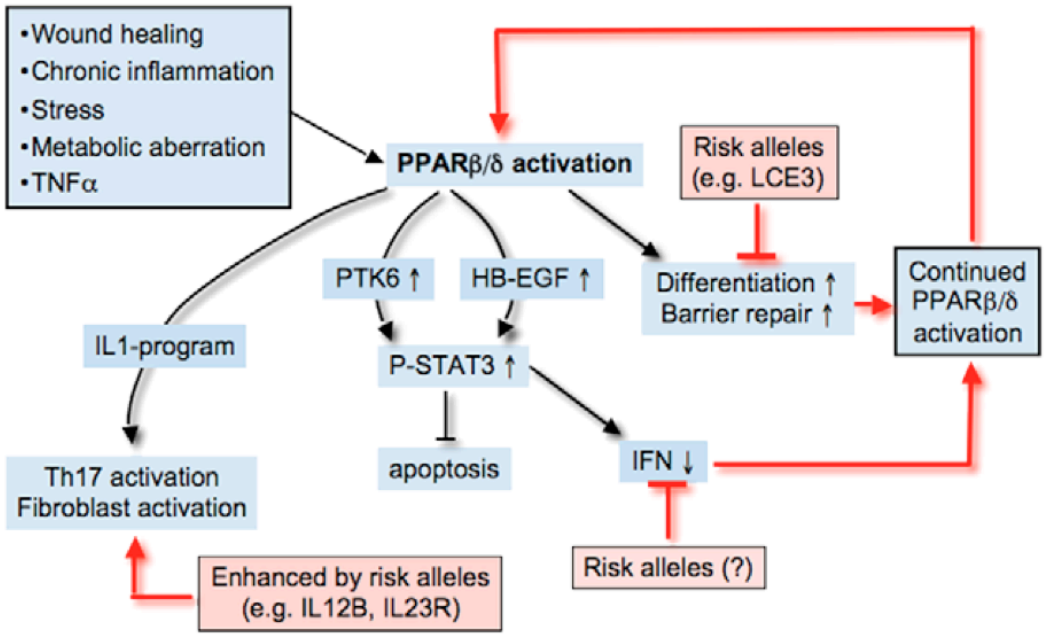

Figure 4.2: Disease Signalling Pathways Mediated By PPARδ. *Schematic diagram showing the PPARδ/STAT3/IL-1 pathway elucidated as a result of this work, showing the role of PPARδ in maintaining psoriasis and the role of the genomic risk alleles. Also shown is a list of factors known to trigger disease flares and also activate PPARδ. Modified from (Romanowska et al 2010).*

4.5 Applicability Of The Current Model For Translational Research

When considering any *in vivo* model of a complex polygenic disease, it is important to remember that in any transgenic system, one can only manipulate a set number of variables within the system. The majority of *in vivo* models are designed so as to allow dissection of the role of particular pathways in a larger system. Given previous data generated by our lab on the role of PPARδ in psoriasis and epidermal development (al Yacoub et al 2008, Romanowska et al 2008), it was important to observe the possible effects of overexpressing PPARδ in an *in vivo* model, to thus obtain a better understanding of the other roles of this protein. The fact that it is the full length human form of the protein expressed in this model adds relevance as it will allow for the development of targeted treatments. Additionally, the system created in this model is fully reversible, thus upon cessation of systemic induction via GW501516 containing diet, the phenotype of the mice returns to normal. This is an important feature, as it not only shows that continuous activation of PPARδ is required to maintain disease activity but also indicates that the model will be responsive to treatments. Therefore, this model exhibits a number of advantages to study pathways and treatments for psoriasis when compared to the human skin graft approaches, as follows. The need for skin biopsies is central to the success of human-skin graft models, which are limited, therefore leading to low throughput. Also, skin graft can take a number of weeks to "settle" after being grafted to the mice. In addition, the mice that are commonly used for such approaches are immunocompromised, which directly contradicts the strong immune component of the disease. In addition, skin-graft approaches only develop some of the features of psoriasis, and usually need a stimulus to achieve this. Therefore, a model that focuses on a central mediator is better suited to study the pathogenesis of psoriasis, such as the

PPARδ model, which as previously discussed, recapitulates many of the key aspects of psoriasis.

4.6 Overlap Between Psoriasis And Metabolic Disease

It is becoming increasingly apparent that there is a significant clinical overlap between psoriasis and metabolic syndrome, which includes obesity, diabetes and cardiovascular disease (Azfar and Gelfand 2008). A patient is considered to have metabolic syndrome if they display at least 3 of the following conditions: abdominal obesity, elevated serum triglycerides, low HDL cholesterol, elevated blood pressure or elevated fasting glucose (Azfar and Gelfand 2008). The clinical features that most often correlate with psoriasis are hypertriglyceridemia and abdominal obesity. Interestingly, obesity may occur before the onset of psoriasis and is considered a risk factor for the disease. In addition, increasing BMI (Body Mass Index) correlates with severity of psoriatic disease (Azfar and Gelfand 2008, Naldi et al 2005). An important feature of psoriasis is the elevation of Th1 cytokines, which chronically is also important in the pathophysiology of metabolic syndrome. On the other hand, inflammatory molecules and hormones produced as a result of obesity, diabetes etc, may also contribute to the pathogenesis of psoriasis. Insulin resistance, a feature that both psoriasis and metabolic syndrome share may also be mediated by inflammatory cytokine produced as a result of either condition, predominantly TNF. It is proposed that TNF may lead to insulin resistance by inhibiting the tyrosine kinase associated with the insulin receptor or via the activation of PPARδ (Azfar and Gelfand 2008, Di-Poi et al 2003, Tan et al 2001). As discussed previously, PPARδ promotes epidermal proliferation, modulates adipogenesis and glucose metabolism, and is highly expressed in psoriatic lesions. PPARδ plays a

role in the fatty acid catabolism in muscle and adipose tissue, further supporting a role in metabolic disease.

4.7 Implications For PPAR Agonists In Metabolic Disease

To date no PPARδ agonists have been approved for clinical use. One clinical study has been performed using GW501516 to examine its effectiveness at treating obesity and symptoms associated with metabolic syndrome. This short-term study, lasting only 2 weeks, found that GW501516 reduced fasting plasma triglycerides, apolipoprotein B, LDL cholesterol, and insulin (Riserus et al 2008). In addition, liver fat content and urinary isoprostanes were also reduced. *In vivo*, weight loss and "leanness" of PPARδ mice being treated with GW501516 compared to control transgenics is also apparent. This could be explained by the other known functions of PPARδ such as fatty acid oxidation. During prolonged periods of treatment with GW501516 (>3 weeks), it is possible that GW501516 is activating endogenous PPARδ in murine tissues and this causes the weight loss observed since the transgene is only expressed in skin. Despite showing clinical effectiveness, treating patients with a PPARδ agonist should be met with caution. Based on the present data, one may speculate that prolonged treatment with GW501516 could precipitate psoriasis as a side effect in genetically predisposed individuals.

4.8 Activation Of STAT3

Aside from the apparent increase STAT3 activation in the skin of induced PPARδ mice, one observation made in this thesis was the differential phosphorylation of two distinct bands as detected by western blot (Figure 3.10), corresponding to two separate isoforms

of STAT3, STAT3α (89 kDa) and STAT3β (80 kDa) (Caldenhoven et al 1996). These isoforms are generated by alternative splicing at exon 23, which results in the deletion of the first 55 nucleotides of exon 23 and results in a frameshift that introduces 7 alternative amino acids followed by a stop codon (Yoo et al 2002). This alternative splicing results in either a full-length isoform (STAT3α), or a truncated isoform lacking the C-terminal activation domain (STAT3β), which respectively have distinct roles in development and inflammation. The truncated isoform of STAT3, despite lacking the C-terminal domain, still retains the phosphorylation site at Tyr705, which is key in its activation (Maritano et al 2004, Yoo et al 2002). Ablation of STAT3α results in mice that survive until 24hr after birth, which suggests that STAT3β partially compensates for the embryonic lethality that arises from complete STAT3 deletion (Maritano et al 2004). It has been suggested that STAT3β can act in a dominant negative respect, however more evidence is emerging that STAT3β may have distinct functions from STAT3α. The differences in phenotype between the two strains of mice can be attributed to the differing roles of the STAT3 isoforms as these mice were generated on the same CB6F1 (C57BL/6 × BALB/cAn) background (Maritano et al 2004). Maritano and colleagues showed that STAT3β is able to induce acute phase genes in the liver of mice challenged with LPS but unable to sustain their induction. Similarly, ablation of STAT3β results in an increase in IL-6 serum levels in response to LPS and in macrophages from these mice results in more TNF and IL-6 produced than compared to control macrophages following treatment with LPS and IFN-γ (Maritano et al 2004, Yoo et al 2002). STAT3α-deficient macrophages on the other hand, produced far less TNF and IL-6 in response to LPS than those cells lacking both isoforms. These data suggest that STAT3α in fact may be involved in the pro-inflammatory changes in PPARδ transgenic mice as well as in psoriasis where differential regulation of both

isoforms has not been examined to date. Thus, it is noteworthy that PPARδ activation induces specific STAT3α phosphorylation.

4.9 Mechanism Of STAT3 Activation

In PPARδ transgenic mice, STAT3 is highly overexpressed, replicating results previously reported for psoriasis (Sano et al 2005). This suggests that STAT3 is a target for PPARδ. However it is not a direct target gene as PPARδ is unable to phosphorylate STAT3 directly as PPARδ possesses no intrinsic tyrosine kinase activity. Previous studies by John Foerster's group revealed that HB-EGF and TGF-α are upregulated in psoriasis (Romanowska et al 2008). These two EGF-family ligands that are upregulated in psoriasis, may be responsible for the phosphorylation and activation of STAT3, which aids in the explanation of why the development of the psoriasis phenotype observed in PPARδ mice is not fully inhibited by the use of the JAK2 inhibitor, WP1066. Another potential kinase that may play a role in the activation of STAT3 is PTK6, which is upregulated in both psoriasis and PPARδ transgenic mice (Romanowska et al 2010). These data suggest that at least two additional kinase pathways are capable of contributing to the activation of STAT3. Finally, in terms of the overlap between psoriasis and metabolic syndrome, it is noteworthy that the leptin receptor can also trigger STAT3 phosphorylation (Maritano et al 2004). Thus, in the context of obesity of lipid derangement increased circulating leptin can converge on the same pathway.

4.10 STAT3 Immunofluorescence

Immunofluorescence protocols were established in the course of this work to examine murine skin samples since the STAT3 antibody available had well documented use in immunofluorescence (Bauer and Patterson 2006, Ernst et al 2009, Tripathi and McTigue 2008). This antibody was also used for western blotting and yielded highly specific results, confirming antibody specificity. For further independent confirmation, we employed the use of a blocking peptide that inhibited the binding of phospho-STAT3 antibody to the antigen. This method was selected as the blocking peptide is highly specific (http://www.cellsignal.com/products/1195.html). In terms of localisation, the localisation of phospho-STAT3 observed in our samples showed localisation to the upper layers of the epidermis with highly specific nuclear staining very consistent with that previously reported for psoriatic lesional skin (Sano et al 2005).

4.11 Inhibition Of STAT3 With WP1066, A Specific JAK2 Antagonist.

Due to the discovery that STAT3 is highly overexpressed and activated in PPARδ mice induced with GW501516, the possibility of inhibiting the activation of STAT3 and the effect of this on the psoriatic phenotype observed in PPARδ mice was investigated. JAK2 is the major tyrosine kinase receptor that phosphorylates STAT3 causing it to be activated in response to stimulation by growth factors and various cytokines. We initially identified a JAK2 inhibitor that is highly specific and had been tested previously on mice of the same genetic background, C57Bl/6j. Thus, we had previously tested this JAK2 antagonist, AG490, but found that it had no detectable effect on the phenotype (unpublished observation), despite this compound being documented as having inhibitory effects *in vitro* (Iwamaru et al 2007, Meydan et al 1996). However,

using the structure of AG490 as a basis, Iwamaru and colleagues developed subsequent compounds that afforded the same inhibitory action observed *in vitro* that would be bioavailable *in vivo*, including WP1066 (Iwamaru et al 2007).

One limiting aspect of STAT3 inhibition is toxicity. Previous studies using the same transgenic mouse strain, C57Bl/6j mice, had shown that mice could tolerate a dosage of 20 mg/kg via intraperitoneal injection; approximately equivalent to 400 µg per mouse (Kong et al 2009), assuming approximate weight is 20g. The dosage selected for the present studies therefore was that of 75 µl injections of 1.25µg/µl WP1066 dissolved in DMSO/PEG 300 (80/20) 3 times per week, resulting in a total weekly dose of almost 300 µg. This dose ensured sub-toxic levels even allowing for variations in weight. Despite all these precautions, after two weeks of treatment, mice began to show signs of toxicity. It appears that the serum concentration of WP1066 may have increased to levels not tolerated by the mice and began to exhibit symptoms of toxicity (Kong et al 2008). However, it does not appear that toxicity caused by WP1066 is responsible for the inhibition of phenotype development because there is partial inhibition of full phenotype observed before the onset of toxicity.

This toxicity caused by JAK2 inhibition is likely due to heightened sensitivity toward apoptosis. Iwamaru and colleagues found that WP1066 treatment of malignant glioma cells with WP1066 resulted in activation of Bax, suppression of c-myc, Bcl-XL and Mcl-1 expression, and induced apoptosis (Iwamaru et al 2007). Similarly, WP1066 treatment was reported to reverse immune tolerance and inhibit cancer cell growth via the induction of apoptosis (Hussain et al 2007). As in the other study, acute and chronic toxicity are observed *in vivo* following increasing doses of WP1066, with mice exhibiting symptoms of diarrhoea, frequent urination and in chronic cases, adhesion of

the mouse intestine to the abdominal wall and muscle (Kong et al 2008). In terms of toxicity development over time, WP1066 treatment was shown to inhibit STAT3 for up to 3 weeks post injection (Iwamaru et al 2007). Importantly, WP1066 is highly specific for JAK2, suggesting that WP1066 related toxicity is unlikely to be mediated by off-target effects (Iwamaru et al 2007).

Although the partial disease reversal confirms a role of STAT3 downstream of PPARδ, it is not the only factor mediating disease development. Firstly, as mentioned, the onset of the psoriasis phenotype is not fully, but only partially inhibited in mice treated with WP1066. Secondly, overexpression of STAT3 alone causes a less widespread psoriasis-like phenotype with a longer latency (Romanowska et al 2010, Sano et al 2005). Despite not being the sole mediator of the psoriasis phenotype in PPARδ transgenic mice, there is no doubt that STAT3 does play a role in the pathogenesis of psoriasis.

4.12 STAT3 And The "Anti Inflammatory Response".

As previously described, the interferon response genes are downregulated in PPARδ mice but upregulated in psoriasis patients. STAT3 was previously shown to cause the same set of transcriptional change, termed "inflammatory response" (Murray, 2006), suggesting that due to its massive overexpression in our model, it may contribute to the downregulation of these genes as well. In line with this, figure 4.3 shows the interferon response genes downregulated in PPARδ mice that have been induced with GW501516 versus control transgenics (white columns). Indeed, downregulation of the most strongly repressed transcript, IFI27, was only partially reversed upon inhibition of STAT3 signalling with WP1066 treatment, confirming that downregulation of IFN-alpha response genes is indeed at least partly mediated by STAT3 activation.

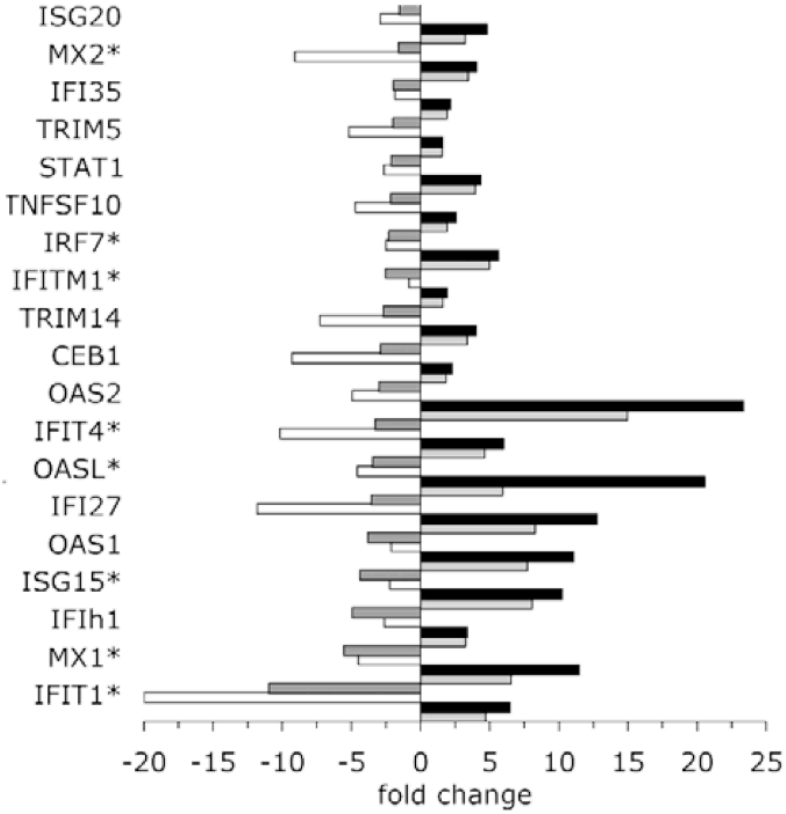

Figure 4.3: Interferon Response Gene Expression In Psoriasis Vs PPARδ Transgenic Mice. *Diagram showing relative expression of interferon response genes in 4 different data sets. Dark grey columns represent data taken from (Dauer et al 2005), white columns represent data from GW501516 fed PPARδ mice vs control treated mice, light grey and black columns represent data from lesional vs. non-lesional skin from psoriasis patients in the GSE14905 and the GAIN datasets. (Modified from (Romanowska et al 2010).*

4.13 Concluding Remarks And Possibilities For Future Work

This thesis details the generation of an *in vivo* model of the human disease psoriasis. These results showed that overexpression of PPARδ, a transcription factor, produced a fully reversible *in vivo* model that reproduces many of the characteristics associated with psoriasis. This model will prove useful in elucidating the signalling pathways that PPARδ regulates. In addition, it will also be useful for the development of a PPARδ targeted treatment for psoriasis. For the full report on this model, please see (Romanowska et al 2010).

Chapter 5: Bibliography

5. Bibliography

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